Final Report P-4488/G-5

METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

FOR

ENERGY, MINES AND RESOURCES, CANADA

BY

J. Christison, Ph.D.
N. Belson
J. Cairns (Consultant)
Environmental and Chemical Engineering Division

Work on this project was conducted under the auspices of the Canada Centre for Mineral and Energy Technology, Energy, Mines and Resources, Canada.

DSS File Number	: 26SQ.23440-3-9138
DSS Contract Serial Number	: OSQ83-00198
CANMET Project Number	: 340212
CANMET Scientific Authority	: Dr. V. Sanmugasunderam

April 26th, 1985

... 21 1986

MICROMEDIA

123 H

METHOD OF ENUMERATION OF T. FERROOXIDANS IN SLURRIES

TABLE OF CONTENTS

1. TITLE PAGE

2. TABLE OF CONTENTS - Phase I - Literature Review
   Phase II - Proposed Programme for Phase III
   Phase IIIA - Evaluation of ATP Assay Applied to T. ferrooxidans Cultures
   Phase IIIB - Release of T. ferrooxidans From Solids and ATP Assessment

3. EXECUTIVE SUMMARY

PAGE

PHASE I

1. INTRODUCTION 2

2. OBJECTIVE 2

3. LITERATURE SEARCH 2

4. GENERAL OVERVIEW 2

5. CELL GROWTH AND METABOLISM IN T. FERROOXIDANS 6
   - 5.1 Carbon Sources 6
   - 5.2 Energy Sources 7
   - 5.3 Nitrogen Sources 8
   - 5.4 Oxygen 8
   - 5.5 Other Chemical Requirements 9
   - 5.6 pH 9
   - 5.7 Temperature 9
   - 5.8 Toxic Effects 10

6. ASSOCIATION OF T. FERROOXIDANS WITH PARTICULATE MATERIAL 11

TABLE OF CONTENTS (Con't)

		PAGE
7.	CURRENT METHODS FOR DIRECT ENUMERATION OF T. FERROOXIDANS	14
7.1	Most Probable Number (MPN) Methods	14
7.1.1	Inaccurate Counts Caused by the Adherence of Bacterial Cells to Particles in the Sample	15
7.1.2	Difficulties in Interpreting a Positive Result	15
7.1.3	Requirement for Long Incubation Periods	15
7.1.4	Toxicity Problems	16
7.1.5	Specificity Problems	16
7.2	Plate Count Methods	17
7.3	Direct Microscopic Counts	19
8.	INDIRECT METHODS FOR ENUMERATION OF T. FERROOXIDANS	20
9.	CONCLUSIONS	22
	BIBLIOGRAPHY	24
PHASE II		36
1.	INTRODUCTION	37
2.	PHASE III PROGRAMME	38
2.1	Phase IIIA - Evaluation of ATP Assay Procedure Applied to T. ferrooxidans Cultures	38
2.2	Phase IIIB - Selection of Test Protocols to Separate T. ferrooxidans Cells From Particle Surfaces	39
PHASE IIIA		40
1.	INTRODUCTION	41
2.	EXPERIMENTAL PROGRAMME	41
2.1	Evaluation of the ATP Assay Procedure Applied to T. ferrooxidans Cultures	41
2.1.1	T. ferrooxidans Strains	41
2.1.2	Selection of ATP Extraction Methodology	42
2.1.3	Modifications to the ATP Extraction Methodology	42

TABLE OF CONTENTS (Con't)

		PAGE
2.2	Adsorption of *T. ferrooxidans* Cells to Particles of Iron Pyrite	44
2.2.1	Adsorption of Cells from Suspensions of Cells in 9K Medium by Percolation Through Columns of Iron Pyrite	44
2.2.2	Effect of Washing Cells Cultured in 9K Medium With Iron-Free Basal Salts Medium Prior to the Adsorption Tests	45
2.3	Attempts to Improve Cell Adsorption by Addition of Yeast Extract	45
3.	RESULTS AND DISCUSSION	48
3.1	*T. ferrooxidans* Strains	48
3.2	ATP Extraction Methodology	48
3.2.1	Background	48
3.2.2	Modifications to ATP Extraction Methodology	49
3.2.3	Adsorption of *T. ferrooxidans* Cells to Particles of Iron Pyrite	50
3.2.4	Effect of Yeast Extract on Adsorption of *T. ferrooxidans* to Iron Pyrite	51
4.	RECOMMENDATIONS FOR FUTURE WORK	58
	APPENDIX I	60
	BIBLIOGRAPHY	61

METHOD OF ENUMERATION OF T. FERROOXIDANS IN SLURRIES

TABLE OF CONTENTS (Con't)

PAGE

PHASE IIIB

1. INTRODUCTION 63

2. OBJECTIVES 63

3. THE ATP ASSAY 64

4. EXPERIMENTAL PROGRAMME 65
   - 4.1 ATP Extraction Methodology Applied to T. ferrooxidans 65
   - 4.1.1 Selection of T. ferrooxidans Stains 65
   - 4.1.2 Development of the ATP Assay Procedure 66
   - 4.1.3 Comparison of the Estimation of Viable Cells by the ATP Assay Procedure and by Protein Measurement 66
   - 4.2 Preparation of T. ferrooxidans Cells Adsorbed on to Sulphide Mineral Particles 66
   - 4.2.1 Background 66
   - 4.2.2 Preparation of T. ferrooxidans Adsorbed to Iron Pyrite 67
   - 4.2.3 Preparation of T. ferrooxidans Adsorbed on Zinc Sulphide Ore 67
   - 4.2.4 Adsorption of Non-Cellular ATP on to Zinc Ore Concentrate 68
   - 4.3 Investigation of Procedures to Release Adsorbed T. ferrooxidans Cells from Zinc Ore Concentrate ... 69
   - 4.3.1 Particle/Cell Substrates for Tests 69
   - 4.3.2 Sampling Procedure 69
   - 4.3.3 Release of Viable Cells from Zinc Ore Concentrate Particles by Chemical/Physical Treatments 69

METHOD OF ENUMERATION OF T. FERROOXIDANS IN SLURRIES

TABLE OF CONTENTS (Con't)

		PAGE
5.	RESULTS AND DISCUSSION	72
	5.1 The ATP Assay Procedure	72
	5.2 Comparison of the ATP and Protein Assay for the Estimation of Viable Cell Numbers	74
	5.3 Preparation of Cell/Particle Materials	77
	5.4 Adsorption of Free (Non-Cellular) ATP by Zinc Concentrate	79
	5.5 Evaluation of Release Treatments	80
	5.5.1 Calculation	80
	5.5.2 Physical Techniques	83
	5.5.3 Physical Treatments Combined with Selected Chemical Reagents	87
6.	CONCLUSIONS	94
7.	RECOMMENDATIONS	97
	APPENDIX I	99
	APPENDIX II	102
	APPENDIX III	104
	APPENDIX IV	106

METHOD FOR THE ENUMERATION OF THIOBACILLUS FERROOXIDANS

EXECUTIVE SUMMARY

1. INTRODUCTION

The Ontario Research Foundation was awarded a contract as a result of an unsolicited proposal, to develop methodology for the direct enumeration of Thiobacillus ferrooxidans in tailings and slurries.

The objective of Phase I of the programme was to critically assess the "state-of-the-art" of enumeration methodologies for T. ferrooxidans. In Phase II, based on the conclusions reached in Phase I, an experimental programme would be developed for subsequent implementation in Phase III.

2. OVERVIEW

Methodologies for the enumeration of microorganisms in clear waters have inherent shortcomings. T. ferrooxidans, the iron-oxidizing, autotrophic bacterium is no exception to the rule. However, due to the tenacious manner with which T. ferrooxidans associates with the surfaces of sulphide-containing particles, major variations have been noted when the "best available" methods are used to enumerate this organism in the presence of sulphide-containing minerals, e.g., tailings, process samples, etc.

3. PROGRAMME

A literature review was undertaken of various enumeration techniques as they apply to the estimation of T. ferrooxidans populations in the presence of particulates. These techniques included most probable number (MPN) procedures, plate count techniques, immunofluorescence procedures and the adenosine triphosphate (ATP) measurement of viable biomass. It was concluded that there is, at present, no satisfactory direct

method to quantitate T. ferrooxidans populations in the presence of particulates. A critical feature for the development of a direct and accurate method of enumeration based on traditional techniques, e.g. plate counts, would be to identify a procedure to release viable cells from their close association with particles. An experimental programme was designed to investigate the efficacy of various physical/chemical treatments for the release of T. ferrooxidans cells tenaciously associated with surfaces of particles. This programme was undertaken in Phase III.

The test materials for the release tests were obtained by culturing a pure strain of T. ferrooxidans on zinc ore concentrate. This approach yielded particulates with bacterial populations adhering to the mineral surfaces greater than 10^8 cells/mL of particulates. Tests with iron pyrite as mineral support were not successful.

The study addressed the important question of how the efficiency of the release of cells can be monitored. A suitable technique must be able to measure viable cell populations associated initially with particles, the percentage of these cells released by a chemical/physical treatment and the retained viability in these cells. Methods for the measurement of viable cells based on culture techniques require long incubation periods and are not applicable to the original particulate materials. A novel approach to the problem was to use the adenosine triphosphate (ATP) assay of viable biomass to estimate the bacterial numbers associated with particles or released into solutions. This non-specific technique could be used to monitor the release of viable bacterial cells provided that defined test materials such as pure T. ferrooxidans cultures associated with zinc ore concentrate were available. By the ATP procedure, the results of release tests could be obtained within a matter of hours instead of weeks as in the case of culture techniques.

The ATP assay procedure developed for this programme was validated by comparing bacterial numbers estimated by this procedure with the values calculated from cellular nitrogen concentrations.

The effects of selected combinations of chemical and physical treatments on the release of T. ferrooxidans from zinc ore concentrate were investigated. The physical techniques included shaking, vortex mixing, ultrasound and homogenization while chemical reagents included selected acids, wetting agents, metal ions, sulphydryl compounds and metabolic inhibitors.

4. CONCLUSIONS AND RECOMMENDATIONS

1. An ATP assay procedure, modified to meet the requirements of the current study, was used to monitor the release of viable cells from particulates.

2. The data for bacterial numbers in T. ferrooxidans liquid cultures, as calculated by the ATP procedure, correlated well with the values estimated from cellular protein concentrations.

3. The ATP assay procedure provided solutions for several of the experimental problems associated with this study. However, to be fully effective as a monitoring technique additional information is required on the correlation between cellular ATP concentrations and cell viability for T. ferrooxidans.

4. Physical techniques/chemical reagents or combinations of them afforded partial release of viable T. ferrooxidans cells from zinc ore concentrate in some instances. However, of the methods tested, none gave an efficient and reproducible release of cells.

5. The tenacity of the association between T. ferrooxidans and particle surfaces appears to be dependent on the celluler loading, the age of cultures, etc. Thus, lower concentrations of cells are more tightly bound and difficult to release.

6. A method remains to be found to efficiently release tightly bound T. ferrooxidans cells from the surfaces of sulphide minerals so that direct traditional culture technique (plate counts) can be used to enumerate the released organisms.

7. Studies should be directed at identifying the precise nature/ mechanism of this tenacious link between T. ferrooxidans and the sulfide minerals.

8. Alternative strategies should be investigated to monitor the biologically-mediated acid-generating potential of environmental samples (tailings etc.). Such approaches should seek methods to measure the acid-producing capabilities of unspecified biological species in tailings samples without requiring the separation of the cells from particles.

METHOD FOR THE ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

PHASE I

Literature Review

METHOD FOR THE ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

1. INTRODUCTION

This report (to end of Phase I) is submitted in accordance with the terms of DSS Contract Serial No. OSQ. 83-00198 and as outlined in the work statement of the unsolicited Proposal # P-4488/G.

2. OBJECTIVE

The objective of the overall programme is to develop a simple and direct protocol for the enumeration of Thiobacillus ferrooxidans in slurries and suspensions of sulphide-containing particles. The objective for Phase I has been to undertake a critical assessment of the state-of-the-art of methods for the enumeration of T. ferrooxidans. Based on our observations, an experimental strategy will be developed (Phase II) in conjunction with CANMET personnel for implementation as Phase III of the total programme.

3. LITERATURE SEARCH

A computer-assisted literature search was done. Key articles and secondary references were then consulted. The references consulted are listed in the bibliography and the supplement. The latter section includes those articles which were reviewed but are not referred to in the text of this report.

4. GENERAL OVERVIEW

T. ferrooxidans is an autotrophic bacterium which has both a beneficial and detrimental role in the mining environment. This unusual situation occurs because of the organism's relatively unique ability to thrive in a low pH environment with a high metallic content and to grow on completely inorganic nutrients. It catalyzes the dissolution of sulphide components of ores by oxidizing both ferrous iron and reduced sulphur compounds to produce acidic ferric sulphate. This reaction is the basis of biological leaching processes for the recovery of metals from sulphide-containing ores. However, when this acidic ferric sulphate solution is generated in sulphide-containing tailings by the action of endogenous organisms, it then contributes to the serious pollution problem of acid mine drainage.

Additional microbial species, such as Thiobacillus thiooxidans and related thiobacilli, also play a role in the biological leaching process and the generation of acid mine drainage. Some organisms appear to have an indirect ecological role whereas the thiobacilli, which are capable of oxidizing reduced sulphur compounds to produce sulphuric acid, play a more direct role. However, T. ferrooxidans remains of particular interest because of the iron-oxidizing ability by which it can initiate and maintain the generation of acidic ferric solutions from the oxidative dissolution of pyrite-containing minerals.

The economic and environmental importance of T. ferrooxidans has stimulated considerable research since its isolation from coal mine drainage waters in 1947 (Colmer and Hinkle, 1947). A series of excellent reviews, which describe the significance of T. ferrooxidans, has been published (Brierley, 1978; Kelly et al., 1979; Torma, 1977; Lundgren et al., 1972). However, much fundamental knowledge about this organism has still to be gained in order to improve the control of both biological leaching processes and acid mine drainage.

There remains significant gaps in the understanding of the precise mechanism by which this microbe can catalyze the dissolution of sulphide minerals (particularly iron pyrite) with the resultant generation of sulphuric acid. Direct attack by the bacteria on mineral sulphides is thought to occur, but this aspect is still a matter for debate. There is a need, therefore, to elucidate further the nature of the interaction of the Thiobacillus with the mineral ore surfaces. Such information will assist the bioengineer to determine the extent to which the cell's physiology might be manipulated in order to optimize the efficiency of biological leaching processes.

The management of tailings for the prevention of acid generation (and the subsequent leaching of toxic metals) would benefit if a method were available to monitor more accurately the changes occurring in the T.ferrooxidans population within the tailings. By the time that acid leachates are observed, the microbial activity within the tailings is well established. It is then more difficult to alleviate the problem. Early-warning techniques are required to permit management personnel to use control measures on tailings, in advance of the observed chemical changes. This should not only be more effective but also economically more attractive to the mining companies.

Related to both the leaching processes and acid mine drainage is the need to develop a further understanding of the microbial ecology of T. ferrooxidans. Evidence has been obtained which suggests a synergistic relationship between T. ferrooxidans and acidophilic heterotrophs (Kingma and Silver, 1981; Arkesteyn and DeBont, 1980). However, there is little knowledge concerning microorganisms which are antagonistic to the growth of T. ferrooxidans. Furthermore, little has been described about the biological mechanisms involved in the successive changes in microbiological populations which eventually lead to a predominance of acidophilic organisms.

Progress in acquiring this key information has been greatly impeded by the inadequate methodology for enumeration of T. ferrooxidans cells in slurries (Brierley, 1978). Nevertheless, many research groups have used procedures such as the most probable number (MPN) technique in order to estimate the magnitude of populations of T.ferrooxidans. In some instances, reservations about the methodology have been noted by the authors (Schubert and Miller, 1982) and a recent study has indicated that the MPN procedure was unreliable when used to enumerate T.ferrooxidans in pyrite-containing tailings (Silver and Sanmugasunderam, 1983). In later sections of this report, we identify why current methods yield inaccurate results and require very long incubation periods. Consequently, until

an improved method is available to enumerate T.ferrooxidans in samples containing particles, studies to assess the effects of genetic, physiological and ecological forces on the behaviour of T.ferrooxidans will be awkward to perform and difficult to interpret.

In this phase we have concentrated on the critical evaluation of the acquired information as it relates to the enumeration of T.ferrooxidans in slurries containing relatively high percentages of particulate material. An effective technique must address two key factors namely:

a) Factors influencing the growth and metabolism of T. ferrooxidans.

b) The nature of the interaction of T. ferrooxidans cells with particles.

These elements will influence, either directly or indirectly, the accuracy of all types of enumeration methods and they will therefore be reviewed in separate sections.

This will be followed by a critical review of the current methods used for the direct enumeration of T. ferrooxidans including most probable number (MPN) counts, plate counts, and direct microscopic counts. Finally, the possible application of indirect methods in direct enumeration technology will be discussed.

In our evaluation we have considered factors such as the techniques for the release/dispersion of T. ferrooxidans adsorbed on to the surfaces of particles and the effect of the presence of other species of acidophilic, iron-oxidizing microorganisms on the accuracy of an enumeration method. Relevant information on these aspects will be noted in the general text.

Secondary factors such as the mineralogy of ores and the effect of ore-processing parameters have also been included but the level of development of the enumeration methodology does not warrant an in-depth review of those secondary factors at this point in time. They will be important when a potential methodology has to be validated with natural samples of differing compositions.

5. CELL GROWTH AND METABOLISM IN T. FERROOXIDANS

T. ferrooxidans is an autotrophic, gram negative, rod-shaped bacterium. The genus Thiobacillus includes organisms distinguished by their ability to derive their energy from the oxidation of reduced sulphur compounds (Buchanan and Gibbons, 1974). Because enumeration methods for viable cells usually require cell growth and/or an active metabolism, some consideration must be given to factors which influence these activities in T. ferrooxidans.

5.1. Carbon Sources

The carbon source for T. ferrooxidans is CO_2, which is fixed via the Calvin pathway (Gale and Beck, 1967). In cultures where CO_2 is in excess, growth is limited by the availability of ferrous iron (as an energy source), while in cultures where the CO_2 level is low, CO_2 becomes the limiting factor for growth. Carbon dioxide, however, is not a requirement for oxidation of ferrous iron, as it has been demonstrated that the oxidation can continue in the absence of CO_2 at a linear rate for at least 140 hours (Kelly and Jones, 1978).

T. ferrooxidans is considered to be a strict autotroph and does not grow on organic sources of carbon. Organic compounds have, in fact, been shown to be inhibitory to the growth of the organism (Tuttle and Dugan, 1976; Murakami et al., 1977). Adaptation of T. ferrooxidans to growth on organic compounds has been reported (Gale and Beck, 1967; Shafia et al., 1972; Tabita and Lundgren, 1971), but these results may be artifacts caused by contamination of the cultures with acidophilic heterotrophs (Guay and Silver, 1975; Silver, 1978).

5.2. Energy Sources

T. ferrooxidans can derive energy by the oxidation of both ferrous ion or reduced sulphur compounds. In this respect, it is unique from other thiobacilli which can only oxidize sulphur compounds (Buchanan and Gibbons, 1974). T. ferrooxidans is capable of growing on elemental sulphur as well as a variety of reduced forms of sulphur such as thiosulphate, tetrathionate, and thiothionate. The organism can be cultured for long periods in sulphur medium and then will adapt to grow in ferrous iron medium. Similarly, the organism is able to adapt to grow on sulphur medium after having been grown on ferrous iron medium (Tuovinen and Kelly, 1974b). Prolonged cultivation, however, on sulphur compounds is facilitated by adding a trace concentration of iron (Eccleston and Kelly, 1978).

While growing on ferrous iron, T. ferrooxidans will tolerate high concentrations of other cations. Thus, it has been suggested that specific binding sites for ferrous ions exist on the cell envelope. (Takakuwa et al., 1977).

In iron-containing medium, the levels of both ferrous and ferric ion appear to be significant. Studies by Kelly and Jones (1978) have indicated that in batch cultures of T. ferrooxidans, the production of ferric ion by bacterial oxidation of ferrous ion can become the main limiting factor to cell growth. This phenomenon of end product inhibition appears to involve a competitive mechanism and it does not affect CO_2 fixation.

It was also found (Kelly and Jones, 1978) that high potassium concentrations can partially offset this inhibition. Other research groups (Yunker et al., 1981; Belyi and Gurevich, 1976), however, overcame this effect by constructing an electrolytic culture vessel in which the ferric ion is converted electrochemically to the ferrous form. This type of reactor prevents the accumulation of the inhibitory

end products and regenerates the supply of ferrous ions for further bacterial energy requirements. Using such a device, Belyi and Gurevich (1976) determined that for optimum growth in batch cultures, ferric ion concentration should be less than 13g/L; the concentration of ferrous ion should be 3 - 5g/L; and the ratio between ferric and ferrous ions should be maintained at the optimum. Kovrov and Denisov (1978) determined with such an electrolytic vessel that the rate of ferrous ion oxidation increased directly with increasing ferrous iron concentration but inversely with total iron (ferrous + ferric) concentration.

5.3. Nitrogen Sources

While nitrogen requirements for T. ferrooxidans are usually supplied by ammonium ions, a strain of this species has also been described which is capable of fixing dinitrogen gas (Kelly and Jones, 1978). Some strains also appear to be capable of using urea (Brierley and Brierley, 1968).

Tuovinen et al. (1979), studied the influence of a variety of nitrogen compounds on ferrous oxidation by two strains of T. ferrooxidans. They found with both strains, that ammonium was a limiting nutrient for ferrous oxidation. One strain was capable of using nitrate, but not nitrite, as a nitrogen source. Seven different amino acids (tested at 0.5mM) either inhibited or had no effect on iron oxidation. Complex nitrogen sources such as beef extract, casein, malt extract, peptone, tryptone, and yeast extract had an inhibiting effect at 0.1 and 0.5%(w/v) but a stimulating effect at 0.005 and 0.01%(w/v) respectively. In the absence of a nitrogen source, ferrous oxidation was linear for at least 160 hours.

5.4. Oxygen

Oxygen is essential to the growth of T. ferrooxidans (Buchanan and Gibbons, 1974). At an optimal pH (approx. 2.5), the limiting oxygen concentration for this bacterium's growth was examined over a range of 25°C to 34°C and found to be approximately 5% of the oxygen concentration at saturation (Myerson, 1981).

5.5. Other Chemical Requirements

Other major nutrient requirements such as phosphorus and sulphate are obtained in the form of inorganic salts (Lazaroff, 1963; Dugan and Lundgren, 1965). Schnaitman et al. (1969) have demonstrated that sulphate requirements can be partially satisfied by phosphate or arsenate ions.

T. ferrooxidans requires a number of trace elements such as potassium and calcium ions but there is also evidence that trace levels of organics may be beneficial. For example, Wakao et al. (1983) observed that the rate of pyrite oxidation was increased many times by the addition of very low quantities (approx. 0.001% w/v)of organic materials. This stimulatory effect was observed even for substances which are not metabolized by T. ferrooxidans. It was therefore suggested that the effect of these materials is to facilitate a physico-chemical interaction of the bacteria and solid particles.

5.6. pH

The growth limits of T. ferrooxidans occur between pH 1-5 (Silverman and Ehrlich, 1964), while the cells are inactivated at pH 9 (Malouf and Prater, 1961). Good growth occurs between pH 1.5 to 3.5 with an optimum of approximately 2.5 (Guay et al., 1975; MacDonald and Clark, 1970). Depending on the nutrient content of the growth medium, the pH optimum may change. For example, growth on thiosulphate is optimal at pH 3.6 (Tuovinen and Kelly, 1974b).

5.7 Temperature

Although the optimum temperature for the growth of T. ferrooxidans is approximately 28^{o}C., the optima for biochemical processes oxidizing metallic sulphates and ferrous iron are higher, ranging up to 35^{o}C. (Silverman and Lundgren, 1959a, 1959b; Guay et al., 1975; MacDonald and Clark, 1970). Razzell (1962) reported that outside a temperature range of 5^{o}C to 37^{o}C., sulfide leaching by T. ferrooxidans is not practical.

5.8. Toxic Effects

As mentioned previously T. ferroxidans is a strict autotroph and is inhibited by the presence of organic materials except at low levels. Brierley (1978) noted that the tolerance of T. ferrooxidans to toxic metals "varies with the strain" although information on the tolerances of the numerous strains was limited. Nevertheless, it can be stated that, compared to many bacteria, T. ferrooxidans appears relatively resistant to toxic metals.

The reaction of a given strain of T. ferrooxidans to metal toxicity will depend on its history and the organism can be adapted to display increased resistance towards metals. Silverman and Ehrlich (1964) reported that freshly isolated cultures of T. ferrooxidans were sensitive to high concentrations of metallic ions but adapted rapidly and became resistant to 5,000 ppm or more of copper, ferrous or ferric iron, aluminum, zinc, molybdenum, cobalt, uranium, and other metals. Other laboratories (Furyaeva et al., 1976; Olson et al., 1981; Sugio et al., 1981; Tuovinen and Kelly, 1974a) have also reported the adaptation of T. ferrooxidans to increased concentrations of heavy metals. Mercury and silver ions are particularly toxic to T. ferrooxidans (Imai et al., 1975) and as little as 5×10^{4} mM Hg has been shown to inhibit this organism (Brierley, 1978). Molybdenum above concentrations of 0.03 mM. (5 mg/L) is inhibitory to the growth of T. ferrooxidans; yet, if this organism behaves in similar manner to T. thiooxidans in sulphur media, trace levels may be stimulatory (Takakuwa et al., 1977).

Anionic detergents, which may be present in samples of mining process effluents from flotation units, have also been shown to be toxic to T. ferrooxidans. Detergents such as sodium dodecyl sulphate and sodium dodecyl benzene sulphonate are inhibitory in the range 10 to 40 ppm (Kleinmann et al., 1981; Garcia et al., 1981).

The presence of particles in a sample may also have an inhibitory effect. Dispirito et al. (1981) observed inhibition of T. ferrooxidans growth at pH 1.5 upon the addition of particles of fluorapatite, glass beads, pyrite, and flowers of sulphur. Except for pyrite, whose inhibitory effect appeared to be caused by a soluble compound, the toxic effect required direct contact with the particles. The authors suggested that bacterial activity at a liquid-solid interface is different than in the bulk liquid phase.

6. ASSOCIATION OF T. FERROOXIDANS WITH PARTICULATE MATERIAL

Association of bacterial cells with particulate matter is a fundamental problem with enumeration techniques. In methods where serial dilutions are required, sampling errors can result due to the heterogeneous nature of the cell-particle suspension. Thus, although a ten-fold dilution of sample may contain one tenth the total number of particles, it may contain almost as many bacteria as the undiluted mixture. This will occur if a few of the particles in the sample have unusually high numbers of cells adhering to them.

If the method is based on dilution of sample to an end point (e.g. the MPN and plate count assays), an additional error occurs at the end point itself. Thus, in a typical MPN assay, there is no differentiation as to whether the highest dilution with demonstrated growth contained originally one cell or one particle with many cells per tube. Similarly, in a plate count, a colony could be formed by a single cell or a group of cells bound together.

Particles may also interfere with the measurement technique itself. For example, in direct microscope counting, particles will obscure the cells, while with turbidity measurements, particles create a false positive signal. Particles may also contain substances which interfere with other types of measurements. An example of this problem is the interference of sulphur particles with the Folin and Ciocalteau reagent for protein measurement (Proteau and Silver, 1977). Finally, as mentioned in Section 5.8, the particles themselves may inhibit the growth of T. ferrooxidans.

From the above discussion, it appears significant to consider in greater detail the current knowledge of the association of T.ferrooxidans cells with particles.

One type of association may involve entrapment. When T. ferrooxidans oxidizes ferrous ions in the presence of sulphate (both required for growth), precipitates which consist of crystalline jarosites and/or amorphous ferric hydroxysulphates are produced (Tuovinen and Carlson, 1979; Lazaroff et al., 1982). When these precipitates form, it is likely that some of the bacterial cells will become trapped within the precipitate.

There is also evidence for a direct interaction of T. ferrooxidans cells with mineral surfaces. Electron microscope studies on pyrite crystals placed in T. ferrooxidans cultures identified etching pits with the shapes of bacterial cells and of chains and clusters of bacterial cells (Bennett and Tributsch, 1978). Another electron microscope study (Berry and Murr, 1978) demonstrated the attachment of T. ferrooxidans cells to ore surfaces and indicated that the attachment occurred specifically at sulphide regions of the ore. This specific attachment also occurred with a Sulfolobus sp. and with T. thiooxidans. In an experiment, which studied the adhesion of T. thiooxidans to sulphur particles, Takakuwa et al. (1979) suggested that the adhesion process involved thiol groups since treatment of the cells with sulphydryl(SH)-binding agents (e.g., p-chloromercuribenzoate or iodoacetamide) prevented adhesion and the blocking could be reversed by the addition of an SH donor such as cysteine. The adhesion process was also apparently energy dependent since it was inhibited by the treatment of cells with metabolic inhibitors (e.g. 2,4-dinitrophenol, 2-heptyl-4-hydroxyquinoline-N-oxide, or sodium azide) or by depriving the cells of oxygen.

Studies involving surfactants have also produced evidence of the cell-surface interaction with T. ferrooxidans. Duncan etal.(1964) found that the presence of surfactants increased leaching rates. Production of a surfactant by T. ferrooxidans has also been reported (Grudev et al., 1978). However, no evidence has been obtained which demonstrates the induction of surfactant synthesis upon exposure of cells to a solid substrate (Tuovinen et al., 1978).

Nevertheless, the extent of the interaction between particles and T. ferrooxidans cells appears to be quite significant. McGoran et al. (1969) estimated that in cultures of T. ferrooxidans in 9K medium, over 97% of the cells were associated with the particulate fraction of the medium, while Grudev et al. (1978) reported that over 80% of the cells in T. ferrooxidans culture had become attached to the mineral particles by the end of logarithmic growth. Adherence to particles can occur quite rapidly. Tuovinen et al. (1983) found that in T. ferrooxidans cell suspensions mixed with particles of pyrite, fluor-apatite, or glass beads, more than 25% of the cells were absorbed within five minutes, while Myerson & Kline (1983) observed that more than 50% of the cells were adsorbed within five minutes on to coal particles or glass beads.

Tuovinen et al. (1983) also noted that the degree of adsorption was independent of pH in the range of pH 1.0 to 4.0, except for glass which had a lower capacity as the pH decreased. Cells killed by ultraviolet light had similar initial adsorption rates, but total adsorption was reduced. Extraction of lipopolysaccharide from the cells had a more pronounced effect on adsorption. Because the experiments were performed with cells which had been washed several times with diluted sulphuric acid, it is unlikely that extracellular organic matter had a significant effect on the adsorption phenomena observed in these studies.

It also appears that the cells are adsorbed quite tenaciously. Myerson and Kline (1983) observed no desorption of T. ferrooxidans cells from coal or glass particles after 48 hours. Increasing the pH (as high as pH 11) also had no effect on desorption. Moreover, Tuovinen et al. (1983) found that repeated washing of T. ferrooxidans cells bound to flowers of sulphur, fluorapatite, glass beads, or pyrite failed to remove the cells. McGoran et al. (1969) noted that the use of mild sonication and surfactants was ineffective in separating the cells from particulates, while LeRoux et al. (1973) reported that shaking (with or without Lubrol W or sodium pyrophosphate, agents found to release bacteria from flocs) reduced rather than increased the estimates of viable cells in populations of T. ferrooxidans.

The literature data confirm that association between T. ferrooxidans cells and particles is significant and occurs under most circumstances. Consequently, this association must be considered in any attempt to improve methodology for the enumeration of T. ferrooxidans.

7. CURRENT METHODS FOR DIRECT ENUMERATION OF T. FERROOXIDANS

7.1. Most Probable Number (MPN) Methods

Most MPN methods for T. ferrooxidans use the 9K medium of Silverman and Lundgren (1959). The literature appears to be devoid of reports concerning improvements over this medium for MPN testing. Freshly prepared medium has a pH of 3.0 to 3.6, contains 9000 ppm ferrous iron as an energy source, ammonium as a nitrogen source, phosphate as a phosphorus source, and also sulphate, potassium, calcium, and magnesium. The carbon source (CO_2) is supplied by the aerobic conditions of incubation and, therefore, shaking decreases the length of incubation requirements.

There are several difficulties with the MPN procedure for T. ferrooxidans and these are discussed in the following sections.

7.1.1 Inaccurate Counts Caused by the Adherence of Bacterial Cells to Particles in the Sample

This problem, which is especially severe in slurries, has been discussed previously in Section 6. It is conceivable that errors of several orders of magnitude could occur because of this factor. To date, little progress has been made towards a solution.

7.1.2 Difficulties in Interpreting a Positive Result

A positive result for growth in the liquid culture medium for T. ferrooxidans is usually assigned when the medium turns the colour characteristic of the bacterial oxidation of ferrous ion to the ferric form. However, with long incubation periods, this endpoint can be somewhat obscured due to oxidation of the ferrous ion by atmospheric oxygen.

In normal culture vessels, an increase in turbidity cannot be used to measure the growth of T. ferrooxidans because the medium itself contains a considerable amount of particles. However, Yunker et al. (1981) were able to measure the growth turbidimetrically by growing the organism in an electrochemical cell which continuously removed ferric precipitates. Such a technique would also prevent the inhibitory effect of ferric ions (Kelly and Jones, 1978). To make such an approach practical as an MPN technique,it would be desirable to have multiple cells constructed. They might appear similar to the cells used in a Bactometer (Bactomatic Inc.), a device which estimates cell numbers by measuring the change in electrical impendance as cells grow in a nutrient medium.

7.1.3 Requirement for Long Incubation Periods

T. ferrooxidans is a relatively slow-growing organism. Generation times ranging from 5.3 to 15 hours (Beck, 1969; Silverman and Lundgren, 1959) have been reported for strains cultivated in the laboratory. However, when tests are conducted on natural isolates rather than laboratory cultures, the former strains will probably not be acclimatized to the environment of the MPN test. It is likely that longer generation times will

occur and there may also be a significant lag period before the organism begins to grow. Thus, an incubation period of almost one week is required before the first indications of an MPN count are provided, while a minimum of three weeks is required to assign a negative (or absent) result to an MPN test.

Clearly, this long incubation period is impractical for use in the process control of leaching operations and it creates difficulties in monitoring the risk of the environmental problems associated with the organism.

7.1.4. Toxicity Problems

When slurries are tested using the MPN method, the lower dilutions of a sample may introduce a significant amount of particulate matter into the test medium. The particles may contain toxic metals which can be leached out as the bacteria begin to grow. Inhibition of bacterial growth by these metals will result in a false low MPN count.

7.1.5. Specificity Problems

Most bacterial species will fail to produce a positive result in media such as 9K because of the low pH, the absence of organic nutrients, and because of their inability to oxidize ferrous iron. However, one exception is the bacterium Leptospirillum ferrooxidans (Norris and Kelly, 1978). This organism does differ from T. ferrooxidans in both its morphology and its inability to oxidize sulphur.

It is noteworthy that other bacteria may also grow in the presence of T. ferrooxidans in 9K medium. For example, there appears to be a close association between heterotrophic, acidophilic bacteria and T. ferrooxidans. This association has been observed even in cultures of T. ferrooxidans which had been thought to be pure (Silver, 1978; Harrison et al., 1980; Guay and Silver, 1975). Thus, Thiobacillus acidophilus was isolated from a culture of T. ferrooxidans by Guay and Silver (1975). It was later demonstrated (Kingma and Silver, 1981) that T. acidophilus can grow on pyruvate, which is excreted by T. ferrooxidans as an inhibitory end product.

Arkesteyn and De Bont (1980) subsequently found that T. acidophilus, which did not grow on ferrous iron medium, could grow to a limited extent in the spent medium of T. ferrooxidans. They also observed that in mixed cultures of the two organisms, the numbers of each organism were almost equal.

Recently, Johnson and Kelso (1983) have found that the contamination problem of acidophilic heterotrophs in T. ferrooxidans cultures may be eliminated by performing a subculture in the presence of high concentrations of copper. The T. ferrooxidans cells are resistant to those conditions whereas the contaminating bacteria are not.

7.2. Plate Count Methods

Plate count techniques for T. ferrooxidans suffer from many of the same problems as MPN testing. They are prone to the same errors caused by adsorption of cells to particulate matter (see Section 6.) and incubation periods are at least as long. On the other hand they are more precise because they do not have the high statistical sampling error which is a characteristic of all MPN tests, and they produce single colonies which can be used to obtain pure cultures of T. ferrooxidans. This is achieved with some difficulty because the cells adhering to inorganic precipitates do not disperse easily (Manning, 1975). However, colony appearance can assist in identifying the organism since only colonies of iron-oxidizing bacteria become first orange and then brown in colour (Manning, 1975).

One of the greatest difficulties in performing a plate count for T. ferrooxidans (in addition to slow growth and problems caused by particulate matter) originates with the solidifying agent. Agar (a galactose polysaccharide), the most commonly used solidifying agent in microbiological media, has been found to inhibit the growth of T. ferrooxidans (Manning, 1975; Tuovinen and Kelly, 1973; Beck, 1967; Bryner and Jameson, 1958).

In an effort to overcome the inhibitory effects of agar, Tuovinen and Kelly (1973) prevented the contact of T. ferrooxidans cells with the agar medium by initially collecting the cells on a membrane filter and then placing the filter on the agar medium. They found that by selecting the optimal pH, agar concentration, and brand of agar, an effective plate count procedure could be obtained. They also demonstrated that galactose (probably present in unpurified agar and produced by acid hydrolysis of the polysaccharide) was inhibitory to T. ferrooxidans.

Since this work, a number of improvements in the plate count method for T. ferrooxidans have occurred. Manning (1975) developed a medium using purified agar which did not require the use of membrane filters. It gave more rapid growth and larger colonies with distinctive morphological characteristics. However, he did not demonstrate that this medium gave high recoveries of the viable cells.

Mishra & Roy (1979) found that either carrageenan (Type 1) or agarose could be substituted for purified agar in Manning's medium. Carrageenan had the advantage that it could be autoclaved with the basal medium (agar had to autoclaved separately or else it would hydrolyze), while agarose was superior in producing large colonies. They also found that the presence of phosphate (0.025% w/v K_2HPO_4) inhibited growth as did agar medium which was prepared with a pH less than 3.0.

Subsequently, Mishra et al. (1983) modified the medium of Manning and using Bapco agar (Japanese), agarose, or carrageenan, they examined the plating efficiency of seven strains of T. ferrooxidans. Their formulation appeared to have excellent storage properties and they also demonstrated good recovery of viable cells. They suggested that any strain of T. ferroxidans could grow on this medium but some adaptation might be required.

A number of laboratories have used silica gel as a solidifying agent, but the preparation of good silica gel plates is tedious and tends to be unpredictable (Manning, 1975). An improved plate count procedure has been reported using membrane filters placed over silica gel plates prepared at pH 1.5 (Yunker et al. 1981)

7.3. Direct Microscopic Counts

The use of direct microscopic counts for estimating T. ferrooxidans numbers has been limited because it is very tedious to distinguish the cells from particles of a similar size. Particles, which are generally present in high of quantities in most samples tested for T. ferrooxidans, also obscure the cells. Furthermore, many other species of bacteria are indistinguishable from T. ferrooxidans when viewed under the microscope.

These problems have been partially overcome with the development of a fluorescent antibody (FA) stain specific for T. ferrooxidans. Cells of T. ferrooxidans treated with this stain are clearly distinguishable when viewed under a microscope equipped with a fluorescent illuminator (Apel et al., 1976). Later Gates and Pham (1979) improved this procedure by developing an indirect fluorescent antibody stain which was simpler to prepare.

Although the fluorescent-antibody (FA) technique permits the visualization of T. ferrooxidans cells, it does not distinguish between dead and living cells. This shortcoming has been solved by Baker and Mills (1982) who combined the FA technique with the INT(2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) technique (i.e FAINT staining) for T. ferrooxidans. With the INT technique, actively metabolizing cells reduce INT to produce a formazan which accumulates as a dark red deposit inside the cell. Thus, after FAINT staining, actively respiring T. ferrooxidans cells would be viewed as fluorescent rods with intracellular formazan deposits. Results can be obtained after an overnight incubation (and potentially within several hours). A comparison of the FAINT method and the MPN test was made and indicated that the MPN test underestimated the number of viable T. ferrooxidans cells by one to three orders of magnitude.

While the FAINT technique appears to be very promising and overcomes the problems of the long incubation periods required for MPN and plate count tests, it does have disadvantages. The antibody preparation specific for T. ferrooxidans is not commercially available and requires animal facilities for production. Compared to typical MPN and plate count techniques, the FAINT technique is labour intensive and requires more skilled personnel.

Slurries also introduce problems. In the presence of large amounts of particles, many of the T.ferrooxidans cells will be obscured from view. Moreover, if the sample contains reducing agents such as ferrous ion, a non-biological reduction of the INT will occur. Some of the particles may also absorb the FA label non-specifically or cause auto-fluorescence problems (in which the particles themselves fluoresce when viewed in the microscope). Reduction of these problems requires sample pre-treatment such as adding bovine serum albumin (BSA); adding rhodamine isothiocyanate-conjugated BSA; or alternatively by washing the sample with a buffer solution. It has been observed, however, that these pre-treatments are not completely satisfactory. Finally, it was also found that biological reduction of INT was significantly slower in acid pH (Baker and Mills, 1982; Gates and Pham, 1979).

In conclusion, there is still a need to improve methodology for direct microscope counting of T. ferrooxidans

8. INDIRECT METHODS FOR ENUMERATION OF T. FERROOXIDANS

The difficulties, described in previous sections, have prompted investigators to estimate populations by correlating T. ferrooxidans cell numbers with various biomass or metabolic indicators. Examples of this approach include measurement of (a) protein content (Proteau and Silver, 1977; Kingma and Silver, 1979), (b) total organic nitrogen (Gormley and Duncan, 1974), (c) radioactive CO_2 fixation (Brierley, 1977), (d) adenosine triphosphate (ATP) content (Tuovinen and Sormunen, 1979), (e) oxygen uptake rate (Brierley, 1978), and (f) iron oxidation rate (Gol'braikht, 1978).

A major problem with the tests which are based on measuring components of biomass, is that they do not distinguish between dead and living cells. These components can also be associated with detrital material. On the other hand, a major concern with tests based on the measurement of metabolic activities is that they cannot distinguish whether a response is generated by a few very active cells or many weakly active cells.

A noteworthy exception to these problems is the ATP (adenosine triphosphate) assay. ATP is found only in living cells (most of a cell's ATP is lost quickly after death) and is not an integral part of non-living systems. Furthermore, it has been found that the amount of ATP in a cell is directly proportional to its mass (Holm-Hansen, 1973). These characteristics, together with the fact that the test is rapid, is easy to perform, and is relatively sensitive (e.g., it is possible to measure as few as 10^3 bacteria in routine testing), make the ATP assay attractive for application to T. ferrooxidans studies. The technique would probably be used more frequently, but difficulties have been encountered with ATP extraction and measurement in the presence of high levels of iron compounds and heavy metals in the samples (Tuovinen and Scrmunen, 1979). Furthermore, the methods, which were described to reduce these problems, were cumbersome and probably somewhat inaccurate, especially when they are compared to more recent improvements in methods for ATP extraction (Afghan et al., 1978).

None of the indirect techniques for estimating the numbers of T. ferrooxidans in a sample, including the ATP assay, measure T.ferrooxidans specifically. Therefore, if T. ferrooxidans is not the major component of the biomass in the sample, false positive results will occur. An approach to overcome this specificity problem is to combine an indirect technique with an enumeration procedure which can provide the required specificity.

An example of this strategy is the FAINT technique, described in Section 7.3., in which the INT assay, an indirect technique to measure microbial dehydrogenase activity, is combined with the fluorescent antibody (FA) technique which is specific for detecting T. ferrooxidans.

Another approach, described by Silverman and Munoz (1979), although not applied to T. ferrooxidans enumeration, is the combination of electrical impedance measurements (a rapid, automated, but not specific, methodology for measuring microbial growth rates) with an MPN technique specific for fecal coliforms.

The above examples demonstrate the potential value of applying indirect methodology in order to improve direct enumeration techniques. It is most probable that this general principle could be further applied to methodology for the enumeration of T. ferrooxidans.

9. CONCLUSIONS

We have undertaken a critical assessment of the current state-of-the-art of enumeration methods for T. ferrooxidans in slurries of particles. This has included a brief review of the various factors in actual samples of slurries or process mixtures, which may affect the growth of the organism, and, therefore, may influence the accurate measurement of the number of viable T. ferrooxidans cells in the samples.

We have identified the major limitations of existing techniques. A key factor, inherent in all direct enumeration methods, is the interference caused by the close association existing between T. ferrooxidans cells and insoluble substrates (sulphides) or other particulate matter. The most probable number (MPN) methods are also limited by difficulties in the interpretation of the MPN endpoint, the long incubation time required, and the possible lack of specificity of the test. Toxic components in samples are also of concern.

Similar weaknesses are identified with plate count techniques. Although there have been improvements with the specificity of the test, other

problems arise from the inhibition of T. ferrooxidans growth by factors in the solidifying agent, e.g. agar. Solutions to this problem have been reported, but their value for the enumeration of unadapted strains of T. ferrooxidans from natural samples has yet to be assessed.

Methods based on microscopic, direct count procedures e.g. the FAINT technique are still sensitive to the presence of particles. They generally require specialized reagents and highly skilled personnel. Their application as routine counting procedures is questionable.

The use of indirect methods such as those based on the measurement of specific metabolites has been reviewed. Their benefit to the current project may be as end-point indicators or to monitor the effect of various test protocols on the viability of T. ferrooxidans cells.

The key factor which must be addressed at the outset of an experimental programme will be the identification of a method for the release of T. ferrooxidans cells from their association with particles. A successful and simple protocol must overcome this initial hurdle.

BIBLIOGRAPHY

Afghan, B.C., R.S. Tobin, and J.F. Ryan. (1978).
"An Improved Method for the Determination of Adenosine Triphosphate in Environmental Samples". Water Res. 12, 783-792.

Apel, W.A., P.R. Dugan, J.A. Filppi and M.S. Rheins. (1976).
"Detection of Thiobacillus ferrooxidans in Acid Mine Environments by Indirect Fluorescent Antibody Staining". Appl. Environ. Microbiol. 32, 159-165.

Arkesteyn, G.J.M.W., and J.A.M. DeBont. (1980).
"Thiobacillus acidophilus : A Study of its Presence in Thiobacillus ferrooxidans Cultures". Can. J. Microbiol. 26, 1057-1065.

Baker, K.H., and A.L. Mills. (1982).
"Determination of the Number of Respiring Thiobacillus ferrooxidans Cells in Water Samples by Using Combined Fluorescent Antibody-2-(p-Iodophenyl)- 3- (p-Nitrophenyl)-5-Phenyltetrazolium Chloride Staining". Appl. Environ. Microbiol. 43, 338-344.

Beck, J.V. (1967).
"The Role of Bacteria in Copper Mining Operations".
Biotechnol. Bioeng. IX, 487-497.

Beck, J.V. (1969).
"Thiobacillus ferrooxidans and its Relation to the Solubilization of Ores of Copper and Iron". In "Fermentation Advances." Edited by D. Perlman. Academic Press, N.Y. pp.747-771.

Belyi, A.V., and Y.L. Gurevich. (1976).
"Optimization of the Culture Medium for Growing Thiobacillus ferrooxidans with Electrochemical Reduction of the Substrate". Upr. Biosint. Vodorodnykh. Bakt. Drugikh. Khemoavtotrofov, Tezisy, Ves. Soveshch. 20-22. (Chem. Abstr. 88, 101405h).

Bennett, J.C., and H. Tributsch. (1978).
"Bacterial Leaching Patterns on Pyrite Crystal Surfaces".
J. Bacteriol. 134, 310-317.

Berry, V.K., and L.E. Murr. (1978).
"Direct Observations of Bacteria and Quantitative Studies of Their Catalytic Role in the Leaching of Low-Grade, Copper-Bearing Waste". In "Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena." Edited by L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press, N.Y. pp.103-136.

Brierley, J.A., and C.L. Brierley. (1968).
"Urea as a Nitrogen Source for Thiobacilli". J. Bacteriol. 96, 573.

Brierley, C.L. (1977).
"Thermophilic Microorganisms in the Extraction of Metals from Ores". Dev. Ind. Microbiol. 18, 273-284.

Brierley, C.L. (1978).
"Bacterial Leaching". Critical Rev. Microbiol. 6, 207-262.

Bryner, L.C., and A.K. Jameson. (1958).
"Microorganisms in Leaching Sulfide Minerals". Appl. Microbiol. 6, 281-287.

Buchanan, R.E., and N.E. Gibbons. (Editors). (1974).
"Bergey's Manual of Determinative Bacteriology". Eighth Edition. The Williams and Wilkins Co., Baltimore, Md.

Colmer, A.R., and M.E. Hinkle. (1947).
"The Role of Microorganisms in Acid Mine Drainage: A Preliminary Report". Science 106, 252-256.

Dispirito, A.A., P.R.Dugan and O.H. Tuovinen. (1981).
"Inhibitory Effects of Particulate Materials in Growing Cultures of Thiobacillus ferrooxidans". Biotech. Bioeng. XXIII, 2761-2769.

Dugan, P.R., and D.G. Lundgren. (1965).
"Energy Supply for the Chemolithotroph Ferrobacillus ferrooxidans". J. Bacteriol. 89, 825-834.

Duncan, D.W., P.C. Trussell and C.C. Walden. (1964).
"Leaching of Chalcopyrite with Thiobacillus ferrooxidans: Effect of Surfactants and Shaking". Appl. Microbiol. 12, 122-126.

Eccleston, M., and D.P. Kelly. (1978).
"Oxidation Kinetics and Chemostat Growth Kinetics of Thiobacillus ferrooxidans on Tetrathionate and Thiosulfate". J. Bacteriol. 134, 718-727.

Furyaeva, A.V., T.I. Pis'man, N.S. Tsechurkin, T.V. Elkina and K.Y. Egamberdieva. (1976).
"Increase in Thiobacillus ferrooxidans Resistance to Copper Ions in a Flow-Type Culture". Upr. Biosint. Vodorodnykh, Bakt. Drugikh. Khemoautotrofov. Tezisy Vses. Soveshch. 103-105. (Chem. Abstr. 88, 101410f).

Gale, L.G., and J.V. Beck. (1967).
"Evidence for the Calvin Cycle and Hexose Monophosphate Pathway in Thiobacillus ferrooxidans". J. Bacteriol. 94, 1052-1060.

Garcia, J.P.M., G.G. Gimenez, E.G. Auban, P.M. Germes and E.H. Gimenez. (1981).
"Growth of Aciduric and Non-aciduric Species of Thiobacillus in Presence of Two Anionic Detergents". Zbl. Bakt. II Abt.2. 136, 682-691.

Gates, J.E., and K.D. Pham. (1979).
"An Indirect Fluorescent Antibody Staining Technique for Determining Population Levels of Thiobacillus ferrooxidans in Acid Mine Drainage Waters". Microbiol. Ecol. 5, 121-127.

Gol'braikht, A.I. (1978).
"Effect of Temperature and Aeration on the Iron-oxidizing Activity of Different Thiobacillus ferrooxidans Cultures". Biol. Zh. Arm. 31, 46-48 (Chem. Abstr. 89, 56202x).

Gormley, L.S., and D.W. Duncan. (1974).
"Estimation of Thiobacillus ferrooxidans Concentrations". Can. J. Microbiol. 20, 1453-1455.

Grudev,S., F. Genchev, and S. Gaidarzhiev. (1978).
"Adhesion of the Bacterium Thiobacillus ferrooxidans to Mineral Surfaces". God. Vissh. Minno.Geol. Inst.Sofia 24, 352-359. (Chem. Abstr. 90, 183075d).

Guay, R., and M. Silver. (1975).
"Thiobacillus acidophilus sp. nov.; Isolation and Some Physiological Characteristics". Can. J. Microbiol. 21, 281-288.

Guay, R., A.E. Torma and M. Silver. (1975).
"Ferrous Ion Oxidation and Uranium Solubilization from a Low Grade Ore by Thiobacillus ferrooxidans". Ann. Microbiol. 126B, 209-219.

Harrison Jr., A.P., B.W. Jarvis and J.L. Johnson. (1980).
"Heterotrophic Bacteria from Cultures of Autotrophic Thiobacillus ferrooxidans: Relationships as Studied by Means of Deoxyribonucleic Acid Homology". J. Bacteriol. 143, 448-454.

Holm-Hansen, O. (1973).
"Determination of Total Microbial Biomass by Measurement of Adenosine Triphosphate". In "Estuarine Microbial Ecology." Edited by L.H. Stevenson and R.R. Colwess. Univ. of South Carolina Press, Columbia.

Imai,K., T. Sugio, T. Tsuchida and T. Tano. (1975).
"Effects of Heavy Metal Ions on the Growth and Iron-oxidizing Activity of Thiobacillus ferrooxidans". Agric. Biol. Chem. 39, 1349-1354.

Johnson, D.B., and W.I. Kelso. (1983).
"Detection of Heterotrophic Contaminants in Cultures of Thiobacillus ferrooxidans and Their Elimination by Subculturing in Media Containing Copper Sulphate". J. Gen. Microbiol. 129, 2969-2972.

Kelly, D.P., P.R. Norris and C.L. Brierley. (1979).
"Microbiological Methods for the Extraction and Recovery of Metals". In "Microbial Technology: Current State, Future Prospects." Edited by A.T. Bull et al. Cambridge University Press. pp.263-308.

Kelly, D.P., and C.A. Jones. (1978).
"Factors Affecting Metabolism and Ferrous Iron Oxidation in Suspensions and Batch Cultures of Thiobacillus ferrooxidans: Relevance to Ferric Leach Solution Regeneration". In "Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena." Edited by L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press, N.Y. pp.19-44.

Kingma Jr.,J.G., and M. Silver. (1979).
Autotrophic Growth of Thiobacillus acidophilus in the Presence of a Surface-active Agent, Tween 80". Appl. Environ. Microbiol. 38, 795-799.

Kingma Jr.,J.G., and M. Silver. (1981).
"Effect of Pyruvate on Growth of Thiobacillus acidophilus". Rev. Can. Biol. 40, 167-172.

Kleinmann, R.L.P., D.A. Crerar and R.R. Pacelli. (1981).
"Biogeochemistry of Acid Mine Drainage and a Method to Control Acid Formation". Min. Eng. 33, 300-305.

Kovrov, B.G., G.V. Denisov and L.G. Sekacheva. (1978).
"Relation of the Rate of Oxidation of Iron(2+) by a Thiobacillus ferrooxidans Culture to Its Concentration". Mikrobiologiya. 47, 400-402. (Chem. Abstr. 89, 125845y).

Lazaroff, N. (1963).
"Sulfate Requirement for Iron Oxidation by Thiobacillus ferrooxidans". J. Bacteriol. 85, 78-83.

Lazaroff, N., W. Sigal and A. Wasserman. (1982).
"Iron Oxidation and Precipitation of Ferric Hydroxysulphates by Resting Thiobacillus ferrooxidans Cells". Appl. Environ. Microbiol. 43, 924-938.

LeRoux, N.W., A.A. North and J.C. Wilson. (1973).
"Bacterial Oxidation of Pyrite". Proc. 10th Intern. Miner. Process. Congr. pp.1051-1066.

Lundgren, D.G., J.R. Vestal and F.R. Tabita. (1972).
"The Microbiology of Mine Drainage Pollution". In Water Pollution Microbiology. Edited by R. Mitchell. J. Wiley and Sons, N.Y. pp.69-88.

MacDonald, D.G., and R.H. Clark. (1970).
"The Oxidation of Aqueous Ferrous Sulphate by Thiobacillus ferrooxidans". Can. J. Chem. Eng. 48, 669-676.

Malouf, E.E., and J.D. Prater. (1961).
"Role of Bacteria in the Alteration of Sulphide Minerals". J. Met. 14, 353-356.

Manning, H.L. (1975).
"New Medium for Isolating Iron-oxidizing and Heterotrophic Acidophilic Bacteria from Acid Mine Drainage". Appl. Microbiol. 30, 1010-1016.

McGoran, C.J., D.W. Duncan and C.C. Walden. (1969).
"Growth of Thiobacillus ferrooxidans on Various Substrates". Can. J. Microbiol. 15, 135-138.

Mishra, A.K., and P. Roy. (1979).
"A Note on the Growth of Thiobacillus ferrooxidans on Solid Medium". J. appl. Bact. 47, 289-292.

Mishra, A.K., P. Roy and S.S.R. Mahapatra. (1983).
"Isolation of Thiobacillus ferrooxidans from Various Habitats and Their Growth Pattern on Solid Media". Curr. Microbiol. 8, 147-152.

Murakami, Y., Y. Kato., J. Someya and Y. Takahara. (1977).
"Some Cultural Characteristics of Iron and Sulphur-oxidizing Bacteria". Hakko Kogaku Kaishi 55, 1-6. (Chem. Abstr. 86, 124700y).

Myerson, A.S. (1981).
"Oxygen Mass Transfer Requirements During the Growth of Thiobacillus ferrooxidans on Iron Pyrite". Biotechnol. Bioeng. XXIII, 1413-1416.

Myerson, A.S., and P. Kline. (1983).
"The Adsorption of Thiobacillus ferrooxidans on Solid Particles". Biotechnol. Bioeng. XXV, 1669-1676.

Norris, P.R., and D.P. Kelly. (1978).
"Dissolution of Pyrite (FeS_2) by Pure and Mixed Cultures of Some Acidophilic Bacteria". FEMS Microbiol. Lett. 4, 143-146.

Olson, J.P., W.P. Iverson and F.E. Brinckman. (1981). "Volatilization of Mercury by Thiobacillus ferrooxidans". Curr. Microbiol. 5, 115-118.

Proteau, G., and M. Silver. (1977).
"A Rapid Method for the Determination of Cellular Protein in the Presence of Elemental Sulphur". Can. J. Microbiol. 23, 1492-1494.

Razzell, W.E. (1962).
"Bacterial Leaching of Metallic Sulphides". Trans. C.I.M. 65, 190-191.

Schnaitman, C.A., M.S. Korczyniski, and D.G. Lundgren. (1969).
"Kinetic Studies of Iron Oxidation by Whole Cells of Ferrobacillus ferrooxidans". J. Bacteriol. 99, 552-577.

Schubert, J.P., and R.M. Miller. (1982).
"Subsurface Oxidation of Pyritic Coal-cleaning Wastes by Chemoautotrophic Bacteria". National Symp. Surface Mining, Hydrology, Sedimentology and Reclamation. University of Kentucky.

Shafia, F., K.R. Brinon, M.W. Heinzman, and J.M. Brady. (1972).
"Transition of Chemolithotroph Ferrobacillus ferrooxidans to Obligate Organotrophy and Metabolic Capabilities of Glucose Grown Cells". J. Bacteriol. 111, 56-65.

Silver, M. (1978).
"Metabolic Mechanisms of Iron-oxidizing Thiobacilli". In "Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena." Edited by L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press, N.Y. pp.3-17.

Silver, M., and V. Sanmugasunderam. (1983).
Personal communication.

Silverman, M., and D.G. Lungdren. (1959a).
"Studies on the Chemolithotrophic Iron Bacteria Ferrobacillus ferrooxidans. I. An Improved Medium and a Harvesting Procedure for Securing High Cell Yields". J. Bacteriol. 77, 642-647.

Silverman, M.P., and D.G. Lungdren. (1959b).
"Studies on the Chemoautotrophic Iron Bacterium Ferrobacillus ferrooxidans: II. Manometric Studies". J. Bacteriol. 78, 326-331.

Silverman, M.P., and H.L. Ehrlich. (1964).
"Microbial Formation and Degradation of Minerals". Adv. Appl. Microbiol. 6, 153-206.

Silverman, M.P., and E.F. Munoz. (1979).
"Automated Electrical Impendance Technique for Rapid Enumeration of Fecal Coliforms in Effluents from Sewage Treatment Plants". Appl. Environ. Microbiol. 37, 521-526.

Sugio, T., T. Tano, and K. Imai. (1981).
"Two Factors Affecting on Iron-oxidizing Activities of Thiobacillus ferrooxidans". Agric. Biol. Chem. 45, 393-403.

Tabita, R., and D.G. Lundgren. (1971).
"Utilization of Glucose and the Effect of Organic Compounds on the Chemolithotroph Thiobacillus ferrooxidans". J. Bacteriol. 108, 328-333.

Takakuwa, S., T. Nishiwaki, K. Hosoda, M. Tominaga and H. Iwasaki. (1977).
"Promoting Effect of Molybdate on the Growth of a Sulphur-oxidizing Bacterium, Thiobacillus thiooxidans". J. Gen. Appl. Microbiol. 23, 163-173.

Takakuwa, S., T. Fujimori, and H. Iwasaki. (1979). "Some Properties of Cell-sulphur Adhesion in Thiobacillus thiooxidans". J. Gen. Appl. Microbiol. 25, 21-29.

Torma, A.E. (1977). "The Role of Thiobacillus ferrooxidans in Hydrometallurgical Processes". Adv. Biochem. Eng. 6, 1-37.

Tuovinen, O.H.,D.P. Kelly, C.S. Dow and M. Eccleston. (1978). "Metabolic Transitions in Cultures of Acidophilic Thiobacilli". In "Metallurgical Applicaions of Bacterial Leaching and Related Microbiological Phenomena." Edited by L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press. N.Y. pp.61-81.

Tuovinen, O.H., and L. Carlson. (1979). "Jarosite in Cultures of Iron-oxidizing Thiobacilli". Geomicrobiol. J. 1, 205-210.

Tuovinen, O.H., and D.P. Kelly. (1973). "Studies on the Growth Thiobacillus ferrooxidans I. Use of Membrane Filters and Ferrous Iron Agar to Determine Viable Numbers, and Comparison with 14 CO2 Fixation and Iron Oxidation as Measures of Growth". Arch. Mikrobiol. 88, 285-298.

Tuovinen, O.H., and D.P. Kelly. (1974a). "Studies on the Growth of Thiobacillus ferrooxidans II. Toxicity of Uranium to Growing Cultures and Tolerance Conferred by Mutation, Other Metal Cations and EDTA". Arch. Microbiol. 95, 153-164.

Tuovinen, O.H. and D.P. Kelly. (1974b). "Studies on the Growth of Thiobacillus ferrooxidans V. Factors Affecting Growth in Liquid Culture and Development of Colonies on Solid Media Containing Inorganic Sulphur Compounds". Arch. Microbiol. 98, 351-364.

Tuovinen, O.H., and P.M. Sormunen. (1979). "A Note on the Determination of ATP by the Luciferin-Luciferase Bioluminescence Reaction in Samples Containing Heavy Metals". Bull. Environ. Contam. Toxicol. 22, 715-716.

Tuovinen, O.H., F.A. Panda and H.M. Tsuchiya. (1979). "Nitrogen Requirement of Iron-oxidizing Thiobacilli for Acidic Ferric Sulphate Regeneration". Appl. Environ. Microbiol. 37, 954-958.

Tuovinen, O.H., P.R. Dugan and A.A. Dispirito. (1983). "Sorption of Thiobacillus ferrooxidans to Particulate Material". Biotechnol. Bioeng. XXV, 1163-1168.

Tuttle, J.H., and P.R. Dugan. (1976). "Inhibition of Growth, Sulphur and Iron Oxidation in Thiobacillus ferrooxidans by Simple Organic Compounds". Can. J. Microbiol 22, 719-729.

Wakao, N., M. Nishina, Y. Sakurai and H. Shiota. (1983). "Bacterial Pyrite Oxidation II. The Effect of Various Organic Substances on Release of Iron from Pyrite by Thiobacillus ferrooxidans". J. Gen. Appl. Microbiol. 29, 177-185.

Yunker, S.B., J.B. Clark, and J.H. Lancaster. (1981). "Cultivation of and Enumeration of Thiobacillus ferrooxidans". Abstracts of the Annual Meeting, ASM - Paper No. I.32

SUPPLEMENT TO BIBLIOGRAPHY

(Papers reviewed but not referred to in text)

Amemiya, K. (1974).
"Relationship Between Growth and Metabolic Activity in the Strict Chemolithotroph, Thiobacillus thiooxidans". Can. J. Microbiol. 20, 1709-1712.

Beck, J.V. (1960).
"A Ferrous Ion Oxidizing Bacterium". J. Bacteriol. 79, 502-509.

Beudeker, R.F., G.C. Cannon, J.G. Kuenen and J.M. Shively. (1980).
"Relations Between D-Ribulose 1,5-Bisphosphate Carboxylase and CO2 Fixing Capacity in the Obligate Chemolithotroph Thiobacillus neopolitanus Grown Under Different Limitations in the Chemostsat". Arch. Microbiol. 124, 185-189.

Beudeker, R.F., J.G. Kuenen and G.A. Codd. (1981).
"Glycollate Metabolism in the Obligate Chemolithotroph Thiobacillus neopolitanus Grown in Continuous Culuture". J. Gen. Microbiol. 126, 337-346.

Beudeker, R.F., J.C Gottschal and J.G. Kuenen. (1982).
"Reactivity Versus Flexibility in Thiobacilli". Antonie. van Leeuwenhoek. 48, 39-51.

Brierley, J.A., P.R. Norris, D.P. Kelly and N.W. LeRoux. (1978).
"Characteristics of a Moderately Thermophilic and Acidophilic Iron-oxidizing Thiobacillus". Eur. J. Appl. Microbiol. 5, 291-299.

Bryant, R.D., K.M. McGroarty, J.W. Costerton and E.J. Laishley. (1983).
"Isolation and Characterization of a New Acidophilic Thiobacillus species (T. albertis)". Can. J. Microbiol. 29, 1159-1170.

Chang, Y.C., and A.S. Myerson. (1982).
"Growth Models of the Continuous Bacterial Leaching of Iron Pyrite by Thiobacillus ferrooxidans". Biotech. Bioeng. XXIV, 889-902.

Cohen,Y., I. de Jonge and J.G. Kuenen. (1979).
"Excretion of Glycollate by Thiobacillus neopolitanus Grown in Continuous Culture". Arch. Microbiol. 122, 189-194.

Dave, S.R., K.A. Natarajan and J.V. Bhat. (1979).
"Microbiological Studies on Thiobacillus ferrooxidans Cultured from Chitradurga Mine Waters". Trans. Indian Inst. Metals. 32, 330-336.

Denison, G.V., B.G. Kovrov and T.F. Kovaleva. (1981).
"Effect of the pH and Temperature of the Medium on the Rate of Oxidation of Fe2+ to Fe3+ by a Culture of Thiobacillus ferrooxidans and the Coefficient of Efficiency of Biosynthesis". Microbiology. 50, 696-700.

Ehrlich, H.L., and S.I. Fox. (1967).
"Environmental Effects on Bacterial Copper Extraction from Low-grade Copper Sulfide Ores". Biotechnol. Bioeng. IX, 471-485.

Gormley, L.S., D.W. Duncan, R.M.R. Brannion and K.L. Pinder. (1975).
"Continuous Culture of Thiobacillus ferrooxidans on a Zinc Sulfide Concentrate". Biotechnol. Bioeng. XVII, 31-49.

Grudev, V.I., K.I. Markov and G.J. Gentchev. (1978).
"Survival of Thiobacillus ferrooxidans After UV Radiation". Comptes rendus de l'Academie Bulgare des Sciences. 31, 599-602.

Guay, R., M. Silver and A.E. Torma. (1976).
"Base Composition of DNA Isolated from Thiobacillus ferrooxidans Grown on Different Substrates". Rev. Can. Biol. 35, 61-67.

Guay, R., and M. Silver. (1980).
"Uranium Biohydrometallurgy". Process Biochem. 15, 8-11.

MacKintosh, M.E. (1978).
"Nitrogen Fixation by Thiobacillus ferrooxidans". J. Gen. Microbiol. 105, 215-218.

Martin, P.A.W., P.R. Dugan and O.H. Tuovinen. (1981).
"Plasmid DNA in Acidophilic, Chemolithotrophic Thiobacilli". Can. J. Microbiol. 27, 850-853.

Milde, K., W. Sand, W. Wolff and E. Bock. (1983).
"Thiobacilli of the Corroded Concrete Walls of the Hamburg Sewer System". J. Gen. Microbiol. 129, 1327-1333.

Murr, L.E., and V.K. Berry. (1976).
"Direct Observations of Selective Attachment of Bacteria on Low-grade Sulphide Ores and Other Mineral Surfaces".
Hydrometallurgy. 2, 11-24.

Murr, L.E., and A.P. Mehta. (1983).
"The Role of Iron in Metal Sulfide Leaching by Galvanic Interaction". Biotechnol. Bioeng. XXV, 1175-1180.

Noike,T., K.Nakamura and J. Matsumoto. (1983).
"Oxidation of Ferrous Iron by Acidophilic Iron-oxidizing Bacteria from a Stream Receiving Acid Mine Drainage". Water Res. 17, 21-27.

Pham, K.D., and J.E. Gates. (1978).
"A Fluorescent Antibody Staining Technique for Enumerating Thiobacillus ferrooxidans in Acid Mine Drainage Waters". Virginia J. Sci. 29, 128.

Pis'man,T.I., K.Y. Egamberdieva, N.S., Pechurkin, A.V., N.S. Furyaeva and T.V. Elking. (1976).
"Oxidation of Iron by Thiobacillus ferrooxidans in a Continuous Culture". Upr. Biosint. Vodorodnykh. Bakt. Drugikh. Khemoavtotrofov, Tezisy Vees. Soveshch., 68-70. (Chem. Abstr. 88, 101390z).

Pozmogova, I., T.V. Pronina and T.A. Petrova. (1982).
"Characteristics of Thiobacillus ferrooxidans Growth in Periodic and Continuous Culture". Izv. Akad. Nauk. SSSR. Ser. Biol. 574-584. (Chem. Abstr. 97, 108501f).

Razzell, W.E., and P.C. Trussell. (1963).
"Isolation and Properties of an Iron-oxidizing Thiobacillus". J. Bacteriol. 85, 595-603.

Rodriguez-Leiva, M., and S. Pichuantes. (1978).
"A Simple Improved Method to Stain Thiobacillus". Can. J. Microbiol. 24, 756-757.

Schaeffer, W.I., and W.W. Umbreit. (1963).
"Phosphotidylinositol as a Wetting Agent in Sulfur Oxidation by Thiobacillus thiooxidans". J. Bacteriol. 85, 492-493.

Silver, M., and O. Dinardo. (1981).
"Factors Affecting Oxidation of Thiosalts by Thiobacilli". Appl. Environ. Microbiol. 41, 1301-1309.

Silverman, M.P. (1967).
"Mechanism of Bacterial Pyrite Oxidation". J. Bacteriol. 94, 1046-1051.

Smith, A.L., and D.P. Kelly. (1979).
"Competition in the Chemostat Between an Obligately and a Facultatively Chemolithotrophic Thiobacillus". J. Gen. Microbiol. 115, 377-384.

Singer, P.C., and W. Stumm. (1970).
"Acid Mine Drainage: the Rate Determining Step". Science 167, 1121-1124.

Sugio,T., T. Tano and K. Imai. (1981).
"Isolation and some Properties of Silver Ion-resistant Iron-oxidizing Bacterium Thiobacillus ferrooxidans". Agric. Biol Chem. 45, 2037-2051.

Sullivan, E.A., J. Zajic and T.R. Jack. (1980).
"Comparison of Biological and Chemical Leaching. Heap Leaching using Thiobacillus ferrooxidans". In "Biogeochemistry of Ancient and Modern Environments." Edited by P.A. Trudinger, M.R. Walter and B.J. Ralph. Springer-Verlag. pp.557-562.

Sullivan, E.A., J.E.Zajic and T.R. Jack. (1980).
"The Effect of Chemical and Biological Redox Reactions on the Growth of Thiobacillus thiooxidans". In "Biogeochemistry of Ancient and Modern Environments." Edited by P.A. Trudinger, M.R. Walter and B.J. Ralph. Springer-Verlag. pp.521-528.

Torma, A.E. (1978).
"Oxidation of Gallium Sulfides by Thiobacillus ferrooxidans". Can. J. Microbiol. 24, 888-891.

Tuovinen, O.H., and D.P. Kelly. (1972).
"Biology of Thiobacillus ferrooxidans in Relation to the Microbial Leaching of Sulfide Ores". Z. Allg. Mikrobiol. 12, 311-346.

Tuovinen, O.H., and D.P. Kelly. (1974).
"Studies on the Growth of Thiobacillus ferrooxidans IV. Influence of Monovalent Metal Cations and Ferrous Iron Oxidation on Uranium Toxicity in Growing Cultures". Arch. Microbiol. 98, 167-174.

Tuovinen, O.H., and D.P. Kelly. (1974).
"Studies on the Growth of Thiobacillus ferrooxidans V. Factors Affecting Growth in Liquid Culture and Development of Colonies on Solid Media Containing Inorganic Sulphur Compounds". Arch. Mikrobiol. 98, 351-364.

Tuovinen,O.H., and D.J.D. Nicholas. (1977).
"Transition of Thiobacillus ferrooxidans KG-4 from Heterotrophic Growth on Glucose to Autotrophic Growth on Ferrous Iron". Arch. Microbiol. 114, 193-195.

Unz, R.F., and D.G. Lundgren. (1961).
"A Comparative Nutritional Study of Three Chemoautotrophic Bacteria: Ferrobacillus ferrooxidans, Thiobacillus ferrooxidans and Thiobacillus thiooxidans". Soil Sci. 92, 302-313.

Yudina, I.N. (1976).
"Activity of a Thiobacillus ferrooxidans Culture in the Presence of Silver and its Compounds". Tr., Tsentr. Nauchno-Issled. Geologorazved. Inst. Tsvetn. Biogorodn. Met. 125, 39-43. (Chem. Abstr. 88, 195173f).

METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

PHASE II

Proposed Experimental Programme

METHOD FOR THE ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

Phase II
Proposed Experimental Programme

1. INTRODUCTION

Based on the observations from the literature review, it is believed that the design of an enumeration method must address two key factors namely:

a) The "neutralization" of the attractive forces involved in the close association between T. ferrooxidans cells and iron pyrite.

b) Selection and modification (if necessary) of a culture technique for direct enumeration.

It is proposed to address only the first factor at the present time.

A concern has been the relatively slow growth rate noted for T. ferrooxidans and the effect it will have on the rate of progress of the experimental progamme. Tests of the efficacy of various reagents and treatments for disrupting the cell/particle interaction must also monitor the effect of the treatment on the viability of the bacterial cells. Routine culture techniques using incubation times of 4-5 days cause a significant extension of the time required to complete the total experimental programme. An approach is proposed to the measurement of cell viability which, if applicable, will circumvent these delays and expedite the experimental programme.

It is proposed that the adenosine triphosphate (ATP) assay procedure be used as a rapid technique for the estimation of cell viability following a treatment applied to cell/particle slurries. This approach will

require a limited amount of laboratory study to validate the use of the ATP technique with T. ferrooxidans. The planned studies are shown in the Phase IIIA programme and they will be in progress while Phase IIIB is being carried out.

A successful ATP assay procedure would assist in the "fine-tuning" of treatment methods, an approach which could not be readily carried out with routine culture techniques.

2. PHASE III PROGRAMME

2.1. Phase IIIA - Evaluation of ATP Assay Procedure Applied to T. ferrooxidans Cultures

Tasks will include:

a) Prepare cultures of 5 strains of T. ferrooxidans.
b) Select a strain which adheres well to iron pyrite particles.
c) Measure the recovery of an ATP standard solution added during the extraction of ATP from T. ferrooxidans cultures in 9K medium.
d) Measure the recovery of an ATP standard solution added during the extraction of ATP from T. ferrooxidans cultures in iron pyrite slurries.
e) Compare ATP recovery from cultures in 9K medium, and also in iron pyrite slurries, with protein measurements on cell biomass of different ages.

The abilities of 4 - 5 strains of T. ferrooxidans to bind to the surface of museum-grade iron pyrite will be compared. By way of the of this series of tests, it is intended to select one strain for use in the remainder of the experimental programme, which not only affords a quantitative recovery of ATP but is also known to associate strongly and rapidly with pyritic surfaces.

2.2. Phase IIIB - Selection of Test Protocols to Separate T.ferrooxidans Cells and Evaluation from Particle Surfaces.

Included in the literature review was an assessment of methods used by various authors to attempt to separate Thiobacillus cells from particles. It is proposed that the scope of the literature search should be expanded to assess methods which have been applied to the separation of other bacteria from particle slurries. Although, the mechanisms of association may not be identical to T. ferrooxidans/pyrite situations, possible approaches may be identified.

Following this limited literature search a list of treatment methods/protocols will be confirmed. It is proposed to test the following variables:

- Sonication
- Homogenization - Waring blendor
- Homogenization - Polytron
- Short term exposure to low pH (approx. 1.0)
- Effect of selected detergents
- Effect of SH neutralizing agents (organic and inorganic).
- Effect of low concentrations of carbon disulphide.
- Effect of high concentrations of copper and possibly other elements.

METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

PHASE IIIA

Evaluation of ATP Assay Applied to *T. ferrooxidans* Cultures

METHOD FOR THE ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

PHASE IIIA

Evaluation of ATP Assay Applied to T. ferrooxidans Cultures

1. INTRODUCTION

The objective of this phase of the programme as outlined in Phase II was to assess the use of the adenosine triphosphate (ATP) assay procedure as applied to Thiobacillus ferrooxidans cultures.

2. EXPERIMENTAL PROGRAMME

2.1 Evaluation of the ATP Assay Procedure Applied to T. ferrooxidans Cultures

2.1.1 T. ferrooxidans Strains

Six strains of T. ferrooxidans were obtained for study in this experimental programme. They are identified as:

1. ATCC 19859	-	American Type Culture Collection
2. Strain PH	-	Dr. A. Harrison, Univ. of Missouri
3. Strain Lp	-	Dr. A. Harrison, Univ. of Missouri
4. Strain ORIG. 1.0	-	Dr. V. Sanmugasunderam, CANMET
5. Strain ORIG. 2.5	-	Dr. V. Sanmugasunderam, CANMET
6. Strain SUBCULTURE	-	Dr. V. Sanmugasunderam, CANMET

The cultures received from Dr. Harrison were pure strains of T. ferrooxidans. The strains were each inoculated into 9K medium (Silverman and Lundgren, 1959) and incubated at 28°C on a rotary shaker (New Brunswick Scientific) at 165 rpm. Each culture was used to inoculate fresh medium as soon as a reddish-brown precipitate began to accumulate in the culture. A lag phase of one to three weeks was noticed in the growth of the respective strains.

2.1.2 Selection of ATP Extraction Methodology

The methodology of Tobin et al. (1978) for the measurement of ATP in sediment samples was selected for the determination of ATP in T. ferroxidans in liquid cultures and T. ferrooxidans associated with particles. This procedure was selected because it minimizes the effects of most of the factors which can interfere with the firefly luciferase assay for ATP.

In the published method, samples are homogenized in a Willems-type homogenizer (Polytron, Brinkmann Instruments Limited) in a mixture of 23% (v/v) chloroform in 0.01M trisodium phosphate buffer, pH 11.7. The particulate matter in the homogenate is removed by centrifugation and filtration. Prior to analysis, the clarified homogenate is diluted with 0.01M glycine buffer pH 10, containing 0.01M ethylenediamine tetraacetic acid (EDTA) as the magnesium salt. The diluted extract is then mixed with a commercial preparation of the enzyme, luciferase (purified from firefly tails), and the resulting luminescence is quantified in a luminescence photometer.

Modifications to the ATP extraction methodology were investigated in order to improve the sensitivity of the technique.

2.1.3 Modifications to the ATP Extraction Methodology

a) Liquid Cultures in 9K Medium

The following modified procedure was developed to the analysis of ATP in T. ferrooxidans cultures in liquid medium.

Samples were first filtered through a glass fibre filter (Whatman, GF/F). The filters with entrapped cells and particles were washed immediately with a solvent such as acetone or ethanol and then homogenized/extracted with only phosphate buffer (0.01M, pH 11.7) using the Polytron homogenizer. Effective disruption of the filter matrix was achieved by cutting the filter in several places before introducing the homogenization. The remainder of the analysis followed the procedure outlined in Section 2.1.2.

The efficiency of the recovery of ATP standard solution spiked into the culture medium was assessed.

b) Pyrite Containing Samples

In order to expedite the current programme, large-scale culturing of T. ferrooxidans cells was not considered for these initial experiments. The use of smaller batches of cells required that the ATP assay method must be able to measure small amounts of biomass, i.e. with sample aliquots of pyrite as low as 0.1mL in volume. The method of Tobin et al.(1978) was not considered to be practical for this situation. The ATP from cells adsorbed to pyrite would be diluted before analysis by a factor of 1000 with a resultant loss in sensitivity.

A further modification of the extraction method was investigated and subsequently introduced into our protocol for the current programme. In this modification, samples were filtered through a small filter unit, treated with solvent, but instead of homogenizing the filter, it was washed twice with 0.5mL of the trisodium phosphate buffer solution to extract of the ATP from the entrapped cells. If necessary, the dilution of the extract with glycine-MgEDTA buffer was also reduced from x10 to x2. Some inhibition of the luciferase was noted with the more concentrated extracts. However, this problem could be compensated for by using the method of additions to quantitate the ATP.

In preparation for a comparison of the more sensitive modified method with the original homogenization procedure, the following test was conducted. An aliquot (9.5mL) of homogenate from a culture of T. ferrooxidans in 9K medium was mixed with 0.5mL of iron pyrite. The mixture was homogenized once more and the processed filtrate was assayed for ATP. The ATP content was not affected by the homogenization in the presence of iron pyrite.

This modified procedure was used to measure the recovery of ATP standards added to pyrite suspensions during extraction.

2.2 Adsorption of T. ferrooxidans Cells to Particles of Iron Pyrite

2.2.1 Adsorption of Cells from Suspensions of Cells in 9K Medium by Percolation Through Columns of Iron Pyrite

In order to compare the efficiency with which T. ferrooxidans cells adsorbed on to the surface of iron pyrite can be desorbed by different treatments, it was necessary that a defined and reproducible substrate be prepared. The goal in this stage of the experimental programme was to prepare iron pyrite particles on to which had been adsorbed cells of T. ferrooxidans. At the same time, the various strains of T. ferrooxidans would be compared for their respective abilities to adsorb on to the pyrite surfaces.

Cultures of strains ATCC 19859, PH and ORIG. 2.5, were prepared in 9K medium. When cell growth was sufficiently high that iron oxidation (as indicated by the colour of the culture liquid) was significant and cells could be observed easily by microscopic examination, (i.e. concentrations greater than 10^7 cells per mL), tests were performed with the cell suspensions in 9K medium. Iron pyrite (100-200 mesh) of a purity similar to museum grade was washed with hot ethanol to disinfect the surfaces. Ethanol was removed from the pyrite by heating overnight at 100°C. Columns of pyrite (1mL aliquots) were prepared and then the respective cell

suspensions (10 mL) were each percolated through a column of pyrite. The filtrate in each case was collected and recycled at least four times through the pyrite column. The contact time was approximately 30 minutes. The treated pyrite from each column was removed and was washed with a 10mL aliquot of fresh 9K medium.

2.2.2 Effect of Washing Cells Cultured in 9K Medium with Iron-free Basal Salts Medium Prior to the Adsorption Tests.

Cultures of two strains, PH and ATCC 19859, were prepared as described earlier. The inorganic precipitates were first removed from each culture liquid by low speed centrifugation. The cells were then recovered by high speed centrifugation and the pellet in each case was washed with the 9K basal salts solution (without ferrous iron). After repeating the wash procedure three times, the pellet of each strain was resuspended in 5mL of the basal salts solution.

The suspensions of washed cells were then passed through columns of pyrite prepared as before. The suspensions were recycled five times. The total elapsed time during these passages was 30 minutes. The treated pyrite in each column was removed and washed gently (three times) to remove unassociated cells. This was achieved by suspending the pyrite in 5mL aliquots of mineral salts medium and letting the particles settle under gravity. The supernatant solution was then decanted and the treatment repeated.

ATP assays were conducted on a portion of the Pyrite exposed to strain PH and also on the first wash liquid.

2.3. Attempts to Improve Cell Adsorption By Addition of Yeast Extract

The above tests yielded poor adsorption of viable cells, as indicated by the ATP assay, to iron pyrite. In order to proceed with the programme, it appeared essential to obtain pyrite slurries with higher concentrations of T. ferrooxidans cells associated with the particles.

Several experiments were conducted to determine if an improved association between T. ferrooxidans and pyrite could be obtained in our laboratory by addition of low levels of organic material to the medium.

In these experiments, cells were washed (prior to their exposure to pyrite) to remove ferrous iron and were then resuspended in a mineral salts medium as described in Section 2.2.2. Two preliminary experiments were conducted in which the washed cells were inoculated into flasks containing 9K basal salts solution. Iron pyrite (5 mL) was then poured into each flask and the flasks were placed on a shaker. Within ten minutes, an aliquot of autoclaved 1% (w/v) yeast extract (YE) solution was added to some of the flasks. After overnight incubation, samples of pyrite were removed, washed three times in sterile basal salts solution, and assayed for ATP.

A more comprehensive experiment using the PH strain of T.ferrooxidans was performed. As in the previous experiments, the cells were first washed in an iron-free medium. However, instead of adding the yeast extract after T. ferrooxidans had been exposed to pyrite, the pyrite and yeast extract were incubated in basal salts solution before inoculation of T. ferrooxidans cells.

The following test conditions were investigated:

(#1): cells inoculated into 50 mL of 9K medium (to ensure that the cells had remained viable after the washing procedure);

(#2): cells inoculated with 50 mL 9K medium to which 5 mL pyrite had been added (to test for toxic effects from the pyrite);

(#3): cells inoculated with 50 mL basal salts solution with no pyrite added (in order to ensure that substances inhibitory to Thiobacillus growth were not present in this medium and also to examine the stability of the culture's ATP level in the absence of an energy source);

(#4): cells inoculated into 50 mL basal salts solution to which 5 mL pyrite had been added (to test for the cells' ability to become associated with pyrite particles).

(#5): cells inoculated into 50 mL basal salts solution to which 5 mL pyrite and 3 mL of 1% (w/v) yeast extract were added 30 min. prior to inoculation (to investigate whether addition of low levels of organic matter facilitates the association of T. ferrooxidans with pyrite particles).

(#6): cells inoculated into 50 mL basal salts solution to which 0.5 mL pyrite and 0.1 mL 1% (w/v) yeast extract had been added 5 min. before inoculation (to examine a test condition with less particulate matter and more similar to that described by Wakao et al. (1983).

Samples were incubated on a rotary shaker at 28^{o}C.

Initially, ATP assays were conducted on the liquid and solid phases after incubation periods of 24 and 48 hours to identify the relative distribution of viable T. ferrooxidans cell mass under the different test conditions. The data indicated significant losses of ATP (within a period of 48 hours) in all mixtures containing pyrite. Therefore, the incubation period was extended and tests were performed on the eighth day.

3. RESULTS AND DISCUSSION

3.1 T. ferrooxidans Strains

Three of the strains (ATCC 19859, ORIG. 2.5 and Strain PH) grew more vigorously and more consistently than the others. Consequently, these strains were utilized in the experimental programme. Strain Lp failed to grow, even after an incubation period of six weeks.

3.2 ATP Extraction Methodology

3.2.1 Background

The methodology of Tobin et al. (1978) was selected because it minimizes the effects of most of the factors which can interfere with the firefly luciferase assay for ATP. For example, metal ions, which can be present at high concentrations in T. ferrooxidans cultures, are precipitated at the highly alkaline pH of the phosphate buffer used for the extraction stage and are then removed during the clarification step. Ions which are not precipitated are chelated by the EDTA in the glycine diluent buffer. Failure to adequately remove these metal ions could otherwise result in either the inhibition of the luciferase enzyme and/or the hydrolysis of extracted ATP before it can be quantitated.

The use of the alkaline pH in the extraction has an added benefit. At this pH value, most of the ionic species present, including the ATP, will be negatively charged. They will tend to repel each other and thus the possibility of ATP loss due to its adsorption on to particulate matter is significantly reduced.

Furthermore, the use of a Willems homogenizer ensures a thorough disruption of the cells and assists the quantitative release of ATP by the cells. While such a vigorous treatment might not be necessary for the samples in this study, it was selected as a good reference protocol against which to assess modifications to the procedure.

3.2.2 Modifications to ATP Extraction Methodology

In adapting the methodology for sediments to the analysis of liquid cultures of T. ferrooxidans, samples were first filtered through a glass fibre filter. The ATP in bacterial cells trapped on the filter can be extracted with smaller volumes of reagents which increases the sensitivity of the analysis. The filtration step also permits, if desired, the cells to be washed prior to extraction of the ATP. It was observed in a preliminary experiment using culture media containing high concentrations of mineral salts, that some co-precipitation of ATP may occur as salts come out of solution in the presence of the chloroform-buffer mixture. Separating the cells from the salt solution by filtration reduced this interference.

The washing of T. ferrooxidans cells (recovered on the glass fibre filter) with a solvent such as acetone or ethanol, prior to the homogenation/extraction with phosphate buffer (no chloroform), afforded a 60% improvement in the extraction of ATP. This was in comparison to the extraction efficiency using the chloroform/phosphate buffer. The reason for this improvement is not known. However, it may have resulted from the prevention of loss of ATP due to salting-out as well as from the reduction of trauma to cells. For instance, since the cells' ATP appears to be fixed immediately by the acetone/ethanol wash, (i.e., the cells' metabolism has been inhibited), the ATP would not be adversely affected during the transfer of filters/cells to glass tubes for homogenization.

The measurement of the efficiency of the recovery of ATP standard solution spiked into the culture medium permits the detection of analytical errors due to problems such as inhibition of the luciferase by constituents of the culture medium, hydrolysis of the ATP, or loss of ATP by adsorption or co-precipitation. Using the modified methodology, the recovery of the ATP spike was close to 100%. Therefore, these factors do not appear to be significant when using this improved extraction method.

The further modification of eliminating the homogenization step and simply extracting the solvent-washed cells with phosphate buffer made it possible to achieve a more sensitive measurement of the ATP from viable cells adsorbed to the surface of pyrite. A more precise comparison of this modified procedure with the homogenization technique has been delayed because of the lack of pyrite samples with high levels of adsorbed T. ferrooxidans. However, recoveries of ATP standard solutions spiked into the pyrite slurries during extraction appeared to be excellent (100%) and in preliminary comparisons with the homogenization approach, the extraction efficiencies were similar.

3.2.3 Adsorption of T. ferrooxidans Cells to Particles of Iron Pyrite

Two laboratories have recently reported rapid and efficient adsorption of T. ferrooxidans cells to particulate matter. DiSpirito et al. (1983) demonstrated that when T. ferrooxidans cells were introduced into suspensions of quartz, pyrite, or fluorapatite, more than 25% of the cells were closely associated with the solid phase within five minutes and approximately 50% within thirty minutes. Similar results were also obtained by Myerson and Kline (1983) on particles of crushed coal and controlled pore glass.

Consequently, in the current programme, problems were not anticipated in obtaining slurries of pyrite particles containing significant numbers of T. ferrooxidans cells adsorbed on to the surfaces. However, this has not been our experience.

Attempts to adsorb T. ferrooxidans cells by filtering cell suspensions in 9K medium through a column of iron pyrite particles were not successful. Microscopic examination of the filtrates and wash solutions indicated that few, if any, of the cells of any of the strains had been retained by the pyrite.

Both DiSpirito et al.(1983) and Myerson and Kline (1983) had washed the T. ferrooxidans cells in iron-free medium prior to testing the efficiency of the adsorption of the cells on to particles. Furthermore, McCready et al. (1983) in a patented process describe a conditioning stage for the T. ferrooxidans cells which includes a similar washing step with iron-free salts medium. They found that this greatly improved the adsorption of cells on to pyritic surfaces. Consequently, an experiment was conducted to determine whether such treatment would improve the level of adsorption which we had observed in the initial tests.

Using the column/percolation approach with limited total contact time (30 min) and T. ferrooxidans cells (strains PH and ATCC 19859) which had been washed in iron-free basal salt medium, a slight improvement was noted in the adsorption of viable cells to iron pyrite. The original eluate of the PH strain cell suspension contained 100ng ATP in the 5mL aliquot, while the 1mL bed of pyrite retained 4.2ng ATP, i.e. only 3% of the total biomass. Although the liquid from the pyrite reacted with strain ATCC 19859 was not analyzed, a similar low amount of ATP (3.7 ng) was also found associated with the pyrite. Therefore, although some association of viable biomass with the pyrite appeared to have been achieved, the efficiency of this association was much less than expected based on the data reported by other laboratories.

3.2.4 Effect of Yeast Extract on Adsorption of T. ferrooxidans to Iron Pyrite

On re-examining the publications of DiSpirito et al. (1983) and Myerson and Kline (1983), it was hypothesized that another possibility for the relatively low adsorption efficiency observed in our laboratory was related to the organic loadings in our system being much less than those of the two other laboratories. For instance, both these laboratories used T. ferrooxidans concentrations of almost 10^{10} cells per mL (calculated from protein measurements), while it is estimated that cell suspensions in the order of 10^{7} cells per mL were employed in our experiments.

Consistent with the concept of a required minimum organic loading for effective association of T. ferrooxidans cells with pyrite is the observation of Wakao et al. (1982), that below a concentration of 10^9 cells per mL, bacterial oxidation of pyrite did not occur. Subsequently, Wakao et al. (1983) reported that addition of a variety of organic substances such as protein, nucleic acid, yeast extract, and peptone dramatically enhanced pyrite oxidation. It was suggested that the organic substances stimulated a physico-chemical interaction between the bacteria and the solid particles.

In a first series of tests, the effect of adding yeast extract to suspensions of iron pyrite and washed T. ferrooxidans cells in iron-free basal salts medium was investigated. When the yeast extract was added 10 minutes after the mixing of cells with iron pyrite, there was only a minor improvement in the adsorption of cells as indicated by the uptake of ATP. Relatively low adsorption efficiencies were still observed and were not improved by increasing the exposure of T. ferrooxidans to pyrite from 30 minutes to an overnight incubation.

In these experiments, it was found that strain PH adsorbed as efficiently as ATCC 19859 and ORIG. 2.5. Because the PH culture was reported to be pure and therefore free of acidophilic heterotrophs, it was selected for exclusive use in future experiments.

The results of a more comprehensive experiment on the effect of yeast extract are summarized in Tables I to III. It was estimated by microscopic examination that, at the beginning of the experiment, each test mixture contained in the order of 10^6 T. ferrooxidans cells per mL.

Washing the cells free of ferrous iron did not appear to damage their viability as demonstrated by the rapid increase in ATP level in 9K medium with no pyrite (test condition #1).

TABLE I

REACTION OF WASHED CELLS OF T. FERROOXIDANS STRAIN PH, WITH ION PYRITE IN DIFFERENT REACTION MIXTURES[1]

Test #	Test Conditions	Total[1] ATP Added (ng)	ATP (ng) Biomass								
			24 h[2]			48 h[2]			8 Days[2]		
			Liquid	Particle	Liquid & Particle	Liquid	Particle	Liquid & Particle	Liquid	Particle	Liquid & Particle
1	Control-Cell Suspension in 9K Medium (50 mL)	55	500		500	2100	-	2100	Not Analyzed		
2	Cell Suspension in 9K Medium (50 mL) + Iron Pyrite (5 mL)	55	3.0	5.0	8.0	6.5	2.5	9.0	19	0.12	19
3	Control - Cell Suspension in Basal Salts Solution (50 mL)	55	50	-	50	80	-	80	18	-	18
4	Cell Suspension in Basal Salts Solution (50 mL) + Iron Pyrite (5 mL)	55	2.5	8.5	11	6.5	2.6	9.0	0.34	0.20	0.54
5	Cell Suspension in Basal Salts Solution (50 mL) + Iron Pyrite (5 mL) + 0.06% (w/v) Yeast Extract	55	5.0	17	22	6.5	34	40	2.1	21	23
6	Cell Suspension in Basal Salts Solution (50 mL) + Iron Pyrite (0.5 mL) + 0.002% (w/v) Yeast Extract	55	46	2.9	49	6.5	Not Analyzed		0.15	2.0	2.2

[1] Based on ATP estimation of viable biomass

[2] Incubation period at 28°C with shaking.

TABLE II

Survival of T. ferrooxidans, Strain PH,
In Liquid Media and in Suspension of Iron Pyrite[1]

Test #	Test Conditions	Relative Amount of Viable Biomass (Liquid Plus Particle Phases)[2]		
		24 h[3]	48 h[3]	8 Days[3]
1	Control - Cell Suspension in 9K Medium (50 mL).	910%	3800%	Not Analyzed
2	Cell Suspension in 9K Medium (50 mL) + Iron Pyrite (5 mL)	15%	16%	35%
3	Control - Cell Suspension in Basal Salts Solution (50 mL).	91%	150%	33%
4	Cell Suspension in Basal Salt Solution (50 mL) + Iron Pyrite (5 mL).	20%	16%	0.98%
5	Cell Suspension in Basal Salt Solution (50 mL) + Iron Pyrite (5 mL) + 0.06% (w/v) Yeast Extract	40%	73%	42%
6	Cell Suspension in Basal Salt Solution (50 mL) + Iron Pyrite (0.5 mL) + 0.002% (w/v) Yeast Extract	89%	Not Analyzed	4.0%

1 Based on ATP measurement of viable biomass and compiled from the data in Table I.

2 Calculated as a percent of the amount of ATP in the flasks at the beginning of the experiment.

3 Incubation period at 28°C with shaking.

TABLE III

Survival of T. ferrooxidans, Strain PH, in Association with Iron Pyrite Particles [1]

Test #	Test Conditions	Relative Amount of Viable Biomass (Particle Phase)[2]		
		24 h[3]	48 h[3]	8 Days[3]
2	Cell Suspension in 9K Medium (50 mL) + Iron Pyrite (5 mL)	9.1%	4.5%	0.22%
4	Cell Suspension in Basal Salts Solution (50 mL) + Iron Pyrite (5 mL)	15%	4.5%	0.36%
5	Cell Suspension in Basal Salts (50 mL) + Iron Pyrite (5 mL) + 0.06% (w/v) yeast extract	31%	62%	38%
6	Cell Suspension in Basal Salts (50 mL) + Iron Pyrite (0.5 mL) + 0.002% (w/v) yeast extract	5.3%	Not Analyzed	3.6%

1 Based on ATP measurement of viable biomass and compiled from the data in Table 1.

2 Calculated as a percent of the amount of ATP in the flasks at the beginning of the experiment.

3 Incubation period at 28°C with shaking.

However, the presence of iron pyrite particles appears to have a significant effect on the levels of viable T. ferrooxidans cells as indicated by ATP assay. Except for test condition #6 in which only one tenth the volume of pyrite was added, significant reductions occurred in the total ATP levels in each flask within the first day of incubation. In test condition #6, where there was less pyrite surface area, a lag in reduction of total ATP levels was noted. This loss of ATP from the liquid phase in the presence of pyrite occurred in both the 9K medium (test condition #2) and the basal salts solution (test condition #4). In all instances, the presence of pyrite caused a significant reduction in the total ATP content (liquid + solid phases) of a test mixture. The addition of yeast extract (test condition #5) appeared to afford some protection to the cells as well as possibly improving the uptake of cells by the pyrite.

When cells were incubated in basal salts solution (test condition #3), a moderate increase was noted in the ATP level at the end of 48 hours. After 8 days at 28^{o}C, there was a decrease in the ATP content of the cell suspension. Our experience has indicated that changes of this magnitude in ATP level can occur in nutrient limited cells over the time interval involved without necessarily a change in cell number. Such changes in ATP content may only reflect a modification of the physiological status of cell.

These preliminary tests have generated somewhat unexpected data. The experiments in Section 2.3 were designed to observe major and relatively rapid changes in the distribution of bacterial cells between particulate and liquid phases. It was not anticipated that such long incubation periods would be monitored or that the losses of ATP would be so significant. Therefore, care is required in interpreting the data obtained to-date, until confirmatory tests have been undertaken.

Nevertheless, the results are important, since to proceed to the major studies of this phase using pyrite/adsorbed cells substrates prepared as above, could generate misleading data. Although the addition of yeast extract appears to permit a significant survival of T. ferrooxidans on pyrite particles over extended periods (see Table III, test #5) the physiological status of the cells adsorbed to the particles requires clarification. For example, the cells do not appear to be multiplying since an overall increase in the ATP levels associated with the particles was not observed during the 8 days of incubation. It is possible that the cells are in some state of sub-lethal shock. If this is the case, the validity of experiments to separate these cells from the particles (i.e. imposing additional stresses on the cells) is indeed questionable.

It should be noted that in the adsorption studies of DiSpirito et al. (1983) and Myerson and Kline (1983), both groups measured the uptake of T. ferrooxidans cells by particles using protein analysis. Since protein is present in both dead and living cells, neither study would establish the viability of the adsorbed biomass.

Similarly, although earlier investigations by DiSpirito et al. (1981) and Tuovinen et al. (1980) demonstrated an inhibition of the oxidation of iron by T. ferrooxidans in the presence of iron pyrite, the cell viability was not measured. The large ATP reductions observed in our programme, suggest that significant death of cells may occur upon unacclimated T. ferrooxidans being brought in contact with high purity iron pyrite (see Table II, test #4). The loss of ATP may reflect the adsorption of cells to the glass surfaces of the flasks - particularly in view of the relatively low cell concentrations used in our test. If the adsorption of cells to the glass surfaces was the explanation for the loss of ATP from liquid phases, it would be reasonable to expect relatively uninterrupted oxidation of iron in the

presence of 9K medium (test condition #2). Since this was not apparent after the 8 day incubation period whereas iron oxidation was visible in Test #2 (no pyrite) within 20 hours, our data appear at present to favour the toxic reaction. However, further experiments are required to confirm this observation.

4. RECOMMENDATIONS FOR FUTURE WORK

It is recommended that future work be directed towards obtaining particles on which T. ferrooxidans cells are actively growing. Our experiments in this programme suggest that this is essential for a meaningful evaluation of separation techniques for the removal of T. ferrooxidans from the particulate matter.

This may be achieved merely by extending the incubation of the test conditions #4, 5, 6 of Table I and, consequently, this experiment is still in progress. For example, Wakao et al. (1983) noted that incubation periods longer than 8 days were required before significant bacterial oxidation of iron from pyrite occurred. Similarly, the deleterious effect of iron pyrite on iron-oxidizing activity of T. ferrooxidans was overcome following an appropriate incubation priod whose length was directly related to the concentration of iron pyrite added to a cell culture (Tuovinen et al. 1981). Inhibition of iron oxidation was observed with 4% (w/v) iron pyrite in T. ferrooxidans cultures (Tuovinen, et al., 1980) and required an acclimation period of up to 20 days.

If necessary, additional experiments will be conducted to acclimate the three strains of the T. ferrooxidans to grow in the presence of iron pyrite and alternative substrates e.g. zinc concentrate. If available, zinc concentrates with varying contents of iron pyrite should be tested for their ability to adsorb the selected strains of T. ferrooxidans. These zinc concentrates, when received from Dr. Sanmugasunderam, will be used to acclimate the bacterial strains before continuing with the experimental programme.

Experiments to monitor the uptake of T. ferrooxidans by pyrite or zinc concentrate will be designed so as to include all variables noted in our initial tests. This will include comparing the uptake of cells as measured by both protein and ATP analyses provided that the protein levels are adequate for measurement. Tests should be conducted to investigate the possible adsorption of cells on to container surfaces.

APPENDIX I

BIBLIOGRAPHY

DiSpirito, A.A., P.R. Dugan, O.H. Tuovinen. (1981). "Inhibitory Effects of Particulate Matter in Growing Cultures of Thiobacillus ferrooxidans". Biotechnol. Bioeng. 23, 2761-2769.

DiSpirito, A.A., P.R. Dugan and O.H. Tuovinen. (1983). "Sorption of Thiobacillus ferrooxidans to Particulate Material". Biotechnol. Bioeng. 25, 1163-1168.

McCready, R.G.L., R.D. Coleman, C.E. Coleman, C.E. Capes, A.G. Kempton and N.A.M. Moneib. (1983). "Bioadsorption Alteration of Iron Sulphide Surfaces". Canadian Patent No. 1145-144.

Myerson, A.S. and P. Kline. (1983). "The Adsorption of Thiobacillus ferrooxidans on Solid Particles". Biotechnol. Bioeng. 25, 1669-1676.

Silverman, M.P. and D.G. Lundgren. (1959). "Studies on the Chemolithotrophic Iron Bacterium Ferrobacillus ferrooxidans. I. An Improved Medium and a Harvesting Procedure for Securing High Cell Yields". J. Bacteriol. 77, 642-647.

Tobin, R.S., J.F. Ryan and B.K. Afghan. (1978). "An Improved Method for the Determination of Adenosine Triphosphate in Environmental Samples". Water Res. 12, 783-792.

Tuovinen, O.H., M.R. Hoffman, F.A. Panda, and H.M. Tsuchiya. (1980). "Inhibition by Pyrite of Bacterial Iron Oxidation Activity". In Biogeochemistry of Ancient and Modern Environments". Edited by P.A. Trudinger, M.R. Walter, and B.J. Ralph. Aust. Acad. Sci/Springer Verlag, New York pp.529-536.

Wakao, N., M. Mishina, Y. Sakurai, and H. Shiota. (1982). "Bacterial Pyrite Oxidation. I. The Effect of Pure and Mixed Cultures of Thiobacillus ferrooxidans and Thiobacillus thiooxidans on Release of Iron". J. Gen. Appl. Microbiol. 28, 331-343.

Wakao, N., M. Mishina, Y. Sakurai and H. Shiota. (1983). "Bacterial Pyrite Oxidation II. The Effect of Various Organic Substances on Release of Iron from Pyrite by Thiobacillus ferrooxidans". J. Gen. Appl. Microbiol. 29, 177-185.

METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

PHASE IIIB

Release of *T. Ferrooxidans* from Solids and ATP Assessment

METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

Phase IIIB
Release of T. ferrooxidans from Solids and ATP Assessment

1. INTRODUCTION

In Phase I, the literature review, it was identified that the most serious factor which prevents the accurate enumeration of T. ferrooxidans in slurries is the organism's tendency to associate tenaciously with the surfaces of sulphide mineral particles. In Phase II, an experimental programme was proposed which would evaluate the efficacy of varius physical/ chemical treatment to release T. ferrooxidans cells associated strongly with the surfaces of particles. This separation of viable cells was considered an essential factor for the development of a direct and accurate enumeration method based on traditional techniques such as plate counts.

To undertake a laboratory investigation of the effect of various treatment protocols on the release of viable cells from particles requires that a monitoring technique for cell viability is available. In Phase II, it was proposed that the adenosine triphosphate (ATP) assay procedure for viable biomass measurement be investigated for use as a rapid monitoring technique of viable cell population associated with particles and of the efficiency of the release of viable cells from particles by the various treatment protocols. In Phase IIIA, the application of the ATP assay to T. ferrooxidans cultures was investigated and modifications to the technique were undertaken to meet the requirements of this programme.

2. OBJECTIVES

In this phase of the programme the objectives were to:

a. Document further the adenosine triphosphate (ATP) assay procedure as applied to T. ferrooxidans associated with particles.

b. Prepare T. ferrooxidans cells adsorbed on the sulphide mineral particles.

c. Undertake laboratory investigations of a method to release adsorbed microorganisms from particulates.

3. THE ATP ASSAY

The ATP assay procedure used in this study was based on the method of Tobin et al.(1978). A brief overview of the methodology is warranted.

ATP is present in all living organisms. The measurement of the ATP value of a sample reflects all active (viable) biomass in the sample and it is not specific for one organism. However, tests have demonstrated a close correlation between the ATP content of a defined bacterial cell suspension and the number of viable cells in the suspension estimated by a traditional counting procedure (e.g., plate count, MPN). By this approach, the mean value for the ATP content of viable bacterial cells such as Escherichia coli has been estimated to be 4.7×10^{-16}g (Malley and Henry, 1981).

ATP is a key intermediate in biological energy metabolism and consequently the physiological status of a cell will influence its ATP content. During the growth cycle of a bacterial cell, the cellular ATP content was shown to vary by a factor of approximately 3.5. (Qureshi and Patel, 1976). Similarly, the growth environment may also alter the cellular ATP content (Lee et al., 1971). Although such variations in the ATP content of living cells can occur, it appears that the ATP content of a cell must be

maintained at a minimum level for survival. It has been demonstrated that ATP is lost from a cell after death (Hamilton and Holm-Hanssen, 1967). However, experiments have suggested that stressed cells, while no longer capable of cell division, may retain significant amounts e.g., 10 - 20%, of the original ATP content. (Chappelle et al., 1977; Cairns et al. 1979).

For the purpose of this study the ATP assay was intended as only a rapid comparative method for evaluating the ability of different chemical agents and/or physical techniques to release viable T. ferrooxidans cells from particles. It would be used to compare the ATP content of cells for which the only variable liable to change the physiological status of the cells (and hence their ATP content) during the course of the experiment would be the physical/chemical treatment to which they had been exposed. The variations noted above would be borne in mind when assessing the data from the experimental studies. More promising release techniques would then be verified by traditional culture techniques for estimating viable cell numbers.

4. EXPERIMENTAL PROGRAMME

4.1 ATP Extraction Methodology Applied to T. ferrooxidans

4.1.1 Selection of T. ferrooxidans Strains

Because ATP is present in all living organisms, its measurement would monitor not only the uptake and release of T. ferrooxidans but also the uptake and release of other species of bacteria present as contaminants. In the literature review for this programme, it was noted that many cultures of T. ferrooxidans, hitherto assumed to be pure, were found to be contaminated with acidophilic heterotrophs. Six strains (including two pure strains of T. ferrooxidans) were compared for their ability to grow in 9K iron-oxidizing medium (Silverman and Lundgren, 1959) and to adsorb and grow on a sulphide mineral particles such as iron pyrite (See Phase IIIA Report). The pure strain (PH) selected for Phase IIIA studies with iron pyrite was also used in the current studies.

4.1.2 Development of the ATP Assay Procedure

Our method to extract and assay ATP from both liquid cultures of T. ferrooxidans and cell/particle suspensions was based on the procedure of Tobin et al.(1978) for the measurement of ATP in sediment samples. The basic methodology was evaluated with both T. ferrooxidans grown in liquid cultures (9K) and T. ferrooxidans adsorbed on to the surface of iron pyrite particles. The original method required some modifications for application to each type of specimen (See Phase IIIA Report).

The eventual ATP extraction and analytical methods used in our study are described in Appendix I.

4.1.3 Comparison of the Estimation of Viable Cells by the ATP Assay Procedure and by Protein Measurement.

Aliquots from a culture of T. ferrooxidans in 9K medium were filtered to recover the cells and the ATP assay was performed as described above. Additional aliquots were filtered and the bacterial cells on the filter were digested with boiling sodium hydroxide solution (1N) as described by Proteau and Silver, (1977). It was found that the solvent extraction stage used by the authors to remove sulphur was not required. The protein content of the extract was determined by the method of Lowry et al.(1967).

4.2. Preparation of T. ferrooxidans Cells Adsorbed on to Sulphide Mineral Particles

4.2.1 Background

Our goal at this stage of the programme was to produce a defined and reproducible substrate to which T. ferrooxidans cells were adsorbed. In the initial series of tests, iron pyrite was selected as the particulate substrate.

4.2.2 Preparation of T. ferrooxidans Adsorbed to Iron Pyrite

Attempts to prepare particle/cell suspensions consisting of T. ferrooxidans, strain PH, adsorbed on to iron pyrite particles were described in the Phase IIIA report. Several acclimation procedures were investigated for their ability to enhance the attachment and growth of T. ferrooxidans on iron pyrite. They included suspending cells in 9K basal salts solution (without iron) or treating iron pyrite particles with yeast extract in the basal salts solution. However, as described in the earlier report, these approaches failed to generate actively growing cultures when treated particles and unacclimated cells were incubated together in basal salts medium.

Growth of T. ferrooxidans was established at a later date in pyrite-containing basal salt medium by modifying the pretreatment conditions. The pyrite was shaken in 1% (w/v) yeast extract solution followed by repetitive washing of the pyrite with the basal salts medium over a period of several weeks. When T. ferrooxidans (strain PH) was inoculated into basal salts containing treated pyrite at 28°C, it began to grow following an initial lag period of 3 weeks. When growth was detected the ATP level on the pyrite particles was 31 ng/mL pyrite whereas the highest ATP concentrations observed previously on pyrite was 6.8 ng/mL.

4.2.3 Preparation of T. ferrooxidans Adsorbed on Zinc Sulphide Ore

Two samples (A and B) of zinc sulphide ore of varying zinc concentration were supplied by Dr. Sanmugasunderam, CANMET. The analytical data forwarded with the samples are shown in Appendix II. The PH strain of T. ferrooxidans, being the pure culture, was again selected for growth experiments. The zinc ore samples were disinfected by incubation in ethanol at 60°C followed by drying at room temperature for several days to evaporate off the solvent. The organism was acclimated to grow on the respective zinc ore samples by sequential transfer and incubation in basal salts medium containing increasing concentrations of the ores to a maximum pulp density of

6.7% (w/v). Where necessary, pH adjustment (to pH 2.1) was used to provide culture conditions favourable for the growth of the organism. The T. ferrooxidans grew well on both ore samples. The Sample A ore concentrate was selected for further studies.

The values for cellular ATP concentrations in T. ferrooxidans cultures (strain PH) in 9K medium and on zinc ore concentrate (recovered from cultures in basal salts medium) were monitored at intervals and compared with the cellular protein values (determined as described in Section 4.1.3.).

4.2.4 Adsorption of Non-cellular ATP on to Zinc Ore Concentrate

In the course of undertaking routine control tests, it was noted that free ATP added to basal salts solution adsorbed on to the zinc ore concentrate. Tests were instituted to confirm that the majority of the ATP on zinc ore particles from T. ferrooxidans was in fact cell-bound. The effects of inhibitors of oxidative phosphorylation or respiration on the ATP levels in liquid cultures of T. ferrooxidans and on particles of zinc ore concentrate/ T. ferrooxidans samples were investigated. The inhibitors selected were 2,4-dinitrophenol and sodium azide. The test samples were incubated in the presence of 900 ppm of the respective inhibitor at room temperature for one hour. The use of heat ($60^{o}C$; 20 min.) to inactivate the bacterial cells was also investigated in a separate test. The temperature chosen, while lethal to T. ferrooxidans cells, was not detrimental to free ATP. In a control experiment, ATP was adsorbed on to the zinc ore particles. The ATP levels in the treated and untreated samples were determined and compared.

As a further confirmation, a 15 tube MPN test (3 dilutions, 5 tubes per dilution) was conducted on the above zinc ore particles washed five times with basal salts medium and serially diluted into 9K medium. These tests indicated that the majority of the ATP associated with the particles was cell-bound.

4.3. Investigation of Procedures to Release Adsorbed T. ferrooxidans Cells from Zinc Ore Concentrate

4.3.1 Particle/Cell Substrates for Tests

Substrates were prepared by culturing T. ferrooxidans (strain PH) in 9K basal salts medium containing 6.7% (w/v) of disinfected zinc ore concentrate. The pulp density, the composition of the basal salts medium, the incubation temperature and the rate of shaking were maintained constant throughout the programme. However, due to time constraints, it was not feasible to arrange for the release tests to be performed on cultures of equal ages. It was also found necessary to increase the size of inoculum for new cultures at the later stages of the experimental programme in order that usable cell/particle subtrates could be obtained in shorter incubation times.

4.3.2 Sampling Procedure

Batches of cell/particles were prepared as needed from active T. ferrooxidans cultures containing zinc ore concentrate. For all release tests or measurements of ATP content on particulates, subsamples (0.1 mL) were measured volumetrically in a cut-off disposable syringe. Thus, the supernatant solution was allowed to drain off and additional slurry was added until the settled volume of wet particulates in the syringe was 0.1 mL. The particulates were then transferred to a centrifuge tube and washed three times with basal salts medium and low speed centrifugation to dislodge loosely bound cells. This was done prior to undertaking the release tests or determining the ATP loading by the routine procedure (Appendix I).

4.3.3 Release of Viable Cells from Zinc Ore Concentrate Particles by Chemical/Physical Treatments

The test protocol used throughout these experiments is described in Appendix III. The washed particle/cell substrates were transferred to tubes and treated with the appropriate reagent and/or physical technique. The ATP contents of the original substrate, the treated particles and the filterable component of the supernatant solution from the treatment were normally each determined. For this screening programme, single analyses were used with the exception of the original starting substrate which, where possible, was analyzed in duplicate.

Within the scope of the current programme, it was only possible to investigate a limited number of treatments and reagents. The work programme was designed to include traditional procedures used elsewhere in the literature as well as to examine alternative approaches to the problem of achieving an effective release of T. ferrooxidans from the surfaces of particles.

Four physical techniques were evaluated including vigorous shaking by hand, vortex mixing, homogenization and sonication. For homogenization the Polytron homogenizer (Brinkmann Instruments) was selected over a Waring Blender largely due to its ease of operation and its compatability with our scale of operation.

The effect of dilute (approximately 0.2N) solutions of hydrochloric, nitric, sulphuric and phosphoric acids was evaluated with and without homogenization. The pH values of the solutions as prepared were 1.14 (sulphuric), 1.67 (phosphoric), 0.95 (hydrochloric) and 0.93 (nitric). Increased ionic strength (1M sodium sulphate) was also used in conjunction with the sulphuric acid solution.

Three detergents and surfactants were selected. We concentrated on the effect of non-ionic and amphoteric compounds. C2M-SF is an amphoteric imidazoline derivative from Miranol Co. The dual positive and negative charges will be present in neutral solutions whereas in acid solutions, the molecule will carry a net negative charge. Igepal CO-630 is a non-ionic, ethoxylated nonyl phenol derivative from Domtar Ltd. Tween 80 (Atlas Chemicals) is a polyoxethylene sorbitan monooleate and is non-ionic.

The effect of these compounds on the release of cellular ATP from zinc ore concentrate was evaluated in combination with physical techniques such as homogenization and sonication. These detergents were also tested in combination with low pH values (pH 1.5) in sulphuric acid (approximately 0.2N).

In view of the similarity between some of the metabolic processes of T. thiooxidans and T. ferrooxidans, we investigated the effect of selected chemical reagents on the release of ATP from zinc ore particle/T. ferrooxidans substrates.

Most of inorganic and organic chemicals were selected for their possible competition with binding sites on either the cells or particles. These compounds are usually toxic to the organism at higher concentrations. In our initial tests we selected concentrations at which the cells were expected to remain viable. Loss of apparent viability in those tests (supernatant + particles) and a poor release of cellular ATP based on the recovered ATP values, would then preclude using higher concentrations of those reagents.

Inorganic cations such as ferric and cupric cations are relatively non-toxic to T. ferrooxidans at lower concentrations, although toxicity does occur as the concentrations are increased. Particles were exposed to 1M ferric and cupric solutions respectively, in conjunction with either homogenization or sonication of the suspensions. The ferric ion was added as both the chloride and nitrate while the copper was present as the sulphate. A pure ferric sulphate was not available.

Organic reagents were selected on the basis of their possible interaction with binding sites on either the substrate or cell. Alternatively, they were chosen for their effect on metabolic processes which may be involved in the binding mechanism. The sulphydryl compounds used were 2-mercaptoethanol, cysteine, glutathione and dithiothreitol.

In our final set of tests we investigated reagents which were potential metabolic inhibitors. The strategy behind this approach was to select reagents whose toxic effect is known to involve reaction with sulphydryl groups (most probably in enzymes) but whose toxic effect is often capable of reversal by adding an excess of a sulphydryl compound such as

cysteine. Our goal was to "shock" the cells with the inhibitor, physically treat the suspensions to release cells, and then immediately "neutralize" the inhibitor. We examined the use of mercury and silver ions and iodoacetamide (0.01M). Tests with T. ferrooxidans cells in 9K medium indicated that after 10 minutes' incubation, at room temperature, the iodoacetamide reaction was reversable. However the mercury and silver inhibited cells were not affected by the addition of excess cysteine. The effect of iodoacetamide (0.01M) on zinc ore particles with adsorbed bacteria was investigated. The test involved incubating washed particles in 4 mL of a 0.01M solution of iodoacetamide for 10 minutes at room temperature. The suspension was sonicated for 15 sec. and 4 mL of 0.5M cysteine solution was immediately added to "neutralize" the inhibitor. After a further 10 minutes' incubation, the samples were centrifuged and the supernatant and particles analyzed for ATP as per usual.

5. RESULTS AND DISCUSSION

5.1 The ATP Assay Procedure

The ATP extraction and assay procedure of Tobin et al. (1978) for the estimation of viable biomass in sediments was adapted to allow the extraction and measurement of cellular ATP from T. ferrooxidans growing in liquid culture medium or adsorbed to particles of iron pyrite and zinc ore concentrate. The modifications increased the sensitivity of the method and permitted the measurement of low concentrations of ATP remaining on particle surfaces.

This extraction procedure was selected for the current programme because it minimizes the effects of most of the potential interfering factors expected to be associated with T. ferrooxidans cultures or sulphide particles. These might cause positive or negative errors with the firefly luciferase assay for ATP. For example, failure to remove or "neutralize" metal ions could result in the inhibition of the luciferase enzyme and/or the hydrolysis of extracted ATP before it could be quantitated. In the above procedure, metal ions are precipitated at the highly alkaline pH value of the phosphate buffer extractant and are removed at the clarification stage. Metal ions which are not precipitated will be complexed by the EDTA in the glycine diluent.

The use of the alkaline extractant, pH 11.7, ensures that most of the ionizable species, including the ATP, will be negatively charged. The possibility of ATP loss by its adsorption on to the surfaces of particles will be significantly reduced.

When a known concentration of ATP was spiked into the 9K culture medium, no interference was noted in the analytical procedure. This indicated that the procedure applied to 9K culture medium was not subject to errors from factors such as the inhibitors of the luciferase in the culture medium, hydrolysis of the ATP, or loss of ATP by adsorption to surfaces or co-precipitation.

When a known amount of ATP was spiked on to a T. ferrooxidans/ iron pyrite mixture during the extraction procedure, the observed recovery of the ATP spike using the improved method was 100%.

During the screening of release procedures, duplicate assays were carried out at regular intervals on both samples of T. ferrooxidans cultures in 9K medium and zinc ore particles recovered from T. ferrooxidans cultures. The range of means of the duplicate tests, the range of coefficient of variation (CV) for these mean values, and the means and standard deviations of the CV values were as follows:

Liquid cultures (9 tests)

Range of Mean Values	: 8.64 - 50.8 ng ATP/mL
Range of CV	: 1.13 - 21.3 ng ATP/mL
Mean CV.	: 8.35 $\pm$ 7.80 ng ATP/mL

Particles (10 tests)

Range of Mean Values	: 102.2 - 4348 ng ATP/mL
Range of CV	: 1.02 - 18.4 ng ATP/mL
Mean CV	: 8.82 $\pm$ 4.75 ng ATP/mL

For the purpose that this procedure was intended, the statistical data were considered to be acceptable. The unexpectedly poorer data for the standard deviation of the liquid cultures reflects the presence of two more extreme values for the CV. They were not, however, outside of the 95% confidence limit and could not be discarded as outliers.

5.2 Comparison of the ATP and Protein Assays for the Estimation of Viable Cell Numbers.

The ATP and protein concentrations of cellular biomass recovered from a liquid culture of T. ferrooxidans in 9K medium are shown in Table I and Figure 1. The data indicate the closest correlations to occur during the "log" phase of the apparent growth cycle.

Cell counts estimated from the ATP and protein data from an 81 hour culture, i.e., close to the end of the log phase of growth, were 1.1×10^{8} and 6.4×10^{8} cells/mL, respectively. These estimations were based on calculated values of 4.7×10^{-16} g ATP/cell (Malley and Henry, 1981) and 2×10^{-14} g protein/cell (Myerson and Kline, 1983). At this sampling point, the respective estimates varied by the smallest factor, i.e., approximately 6. In view of the different values quoted by other researchers for the mean ATP and protein contents of bacterial cells, this deviation was not considered significant. With the exception of the 251 hour culture which showed the respective estimates of bacterial cells to differ by a factor of 20, the remaining samples at different stages in the growth cycle differed by a factor of approximately 10.

The data suggest that a reasonable correlation may be expected with younger cultures but as cells age or enter a dormant stage, the estimated cell numbers calculated from protein data may be expected to differ from the ATP derived number by a greater factor. This may reflect variations in the cellular composition and/or level of metabolic activity. The data are too limited to draw more specific conclusions.

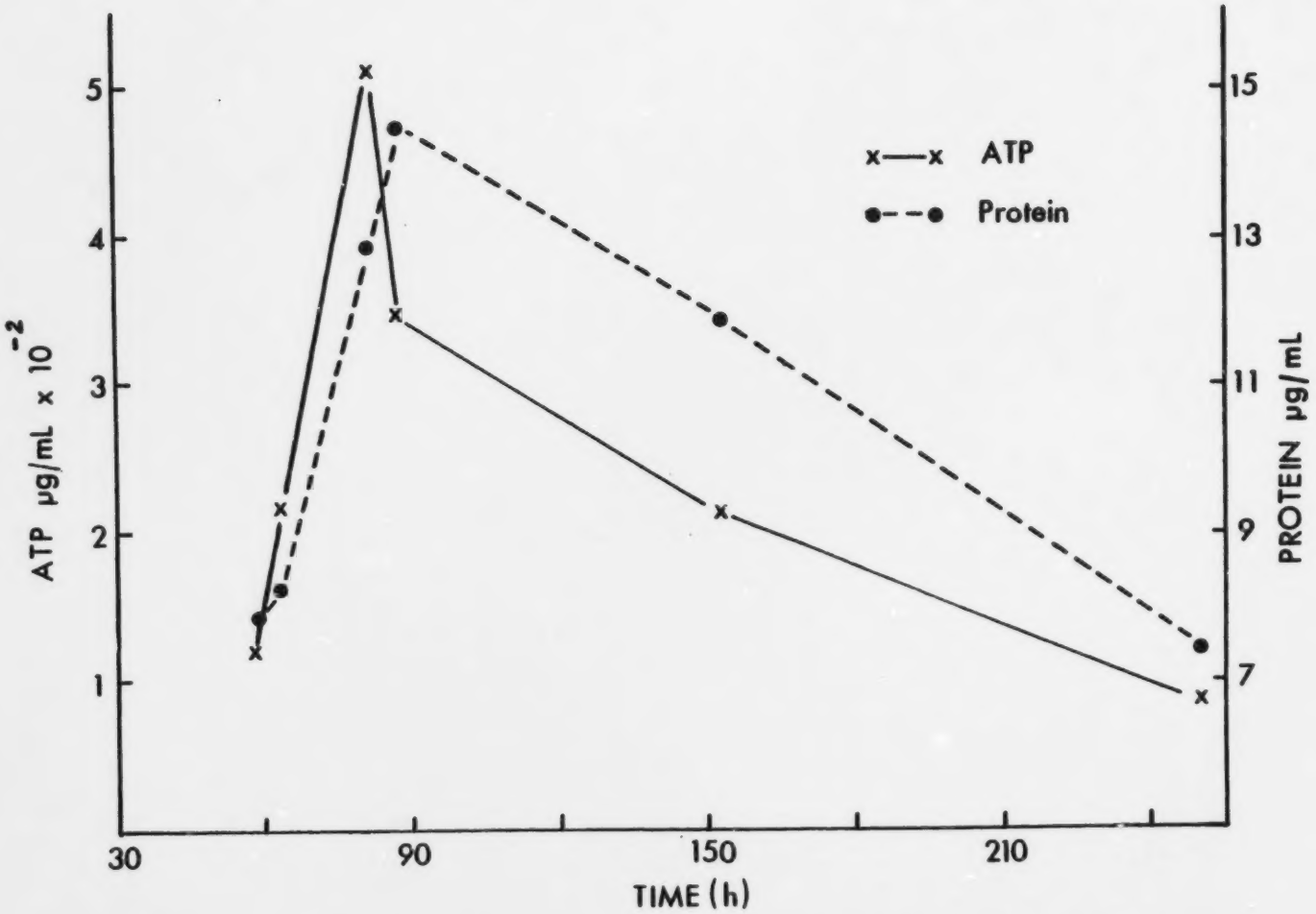

Figure 1 Comparison of the ATP and Protein Assay Techniques for Monitoring the Growth of T. ferrooxidans in 9K Medium

TABLE 1: COMPARISON OF BACTERIAL PROTEIN AND ATP VALUES IN A LIQUID CULTURE OF T. FERROOXIDANS, STRAIN PH[1]

INCUBATION TIME (h)	BACTERIAL PROTEIN µg/mL	BACTERIAL ATP µg/mL	PROTEIN / ATP
48.5	7.83	1.18×10^{-2}	6.6×10^{2}
63	8.24	2.16×10^{-2}	3.8×10^{2}
81	12.9	5.08×10^{-2}	2.5×10^{2}
87	14.5	3.45×10^{-2}	4.2×10^{2}
153	11.8	2.13×10^{-2}	5.6×10^{2}
251[2]	7.42	8.64×10^{-3}	8.6×10^{2}

[1] 9K medium

[2] Values for 250 h are taken from a different but matching culture

Compared with the above correlations in liquid cultures, the cell numbers on zinc ore particulates (Table II) estimated by the two assay techniques deviated by a greater margin with the protein derived value again being higher. In samples analyzed from both younger (17 days) and older (34 - 40 days) cultures, cell estimates by the respective methods differed by orders of magnitude as high as $10^2 - 10^3$. Subsequent tests have indicated that the measured values for protein concentration on the particles are subject to a positive error. Kjeldahl nitrogen analyses of sodium hydroxide extracts were compared with protein values on the same extracts determined by the Lowry procedure. The protein content estimated from the former technique was significantly lower (by several orders of magnitude). The discrepancy is attributed to an analytical error. The precise component(s) from the zinc sulphide particles responsible for the positive bias was not determined. However, it did not appear to originate from sulphur since the extraction with benzene according to Proteau and Silver (1977) had no effect on the protein value analyzed by the Lowry procedure. Some metal ions are known to cause positive errors and this is the most probable explanation for the deviation.

5.3 Preparation of Cell/Particle Materials

In view of the lack of specificity of the ATP assay for T. ferrooxidans, a defined substrate consisting of T. ferrooxidans adsorbed on to a suitable sulphide mineral was prepared for this series of experiments. The substrate was obtained by acclimating T. ferrooxidans, Strain PH (a pure strain of the organism made available to us by Dr. A. Harrison, University of Missouri) to grow on zinc ore concentrate, sample A, in 9K basal salts medium (without added iron).

In growing cultures, the organism associated with the particle surfaces as indicated by both the ATP and protein concentrations on particles recovered from active cultures. Based on these ATP and protein values (Table II), populations of viable cells greater than 10^8 cells per mL of particles were closely associated with the particles in older cultures.

TABLE II: COMPARISON OF PROTEIN AND ATP VALUES ADSORBED ON TO PARTICLES OF ZINC ORE CONCENTRATE IN T. FERROOXIDANS CULTURES

AGE OF CULTURE (h)	CULTURE NO.	PROTEIN µg/mL PARTICLES	ATP µg/mL PARTICLES	PROTEIN / ATP	BACTERIAL CELL NO.	
					FROM[1] PROTEIN	FROM[2] ATP
394	1	3.36×10^{5}	1.94	1.7×10^{5}	1.7×10^{13}	4.1×10^{9}
806	2	2.24×10^{5}	1.06	2.1×10^{5}	1.1×10^{13}	4.5×10^{9}
950	2	1.99×10^{5}	4.35	4.6×10^{4}	9.9×10^{12}	9.3×10^{9}

[1] Based on a value of 2×10^{-8} µg protein/cell (Myerson and Kline, 1983)

[2] Based on value of 4.7×10^{-10} µg ATP/cell (Malley and Henry, 1981)

Particles recovered from cultures were washed to remove loosely bound cells. Following this treatment, the highest ATP concentration on samples of zinc ore concentrate recovered from T. ferrooxidans cultures was 4348 ng ATP/mL of ore. This was equivalent to approximately 10^{16} cells/mL of ore and was obtained from a culture after an incubation period of 45 days.

The highest ATP concentrations on zinc ore concentrate were detected in older cultures. The protein/ATP ratios for particulates from older cultures were also very high and in our release tests, differences were noted in the results of tests performed with T. ferrooxidans adsorbed on zinc ore concentrate from young and old cultures.

The higher concentrations of cell-associated ATP on zinc ore particles also permitted processed solutions from the particles to be sequentially diluted to ensure that errors in the ATP assay from potential enzyme inhibitors such as heavy metals or chemical residues would be minimized.

5.4 Adsorption of Free (Non-cellular) ATP by Zinc Concentrate

The observation that free ATP in solution could quantitatively bind to the surface of zinc ore particles made it necessary to undertake further characterization of the zinc ore/ T. ferrooxidans substrate. It was found that within a contact time of one hour, 0.8mL of zinc concentrate quantitatively bound 100ng of ATP from the basal salts medium. This adsorbed ATP was relatively stable. Almost 24 ng remained on the particles after 24 hours' incubation at room temperature. In earlier tests with iron pyrite, the chemisorption of free ATP had been significantly less (approximately 8ng/mL particles) than with the zinc ore concentrate. This difference may have been due solely to a surface area factor since the particle size of the zinc ore was less then 200 mesh compared with 100-200 mesh in the case of the iron pyrite. The ATP adsorbed on the zinc concentrate was extracted into the alkaline phosphate buffer during the ATP assay procedure. Of concern, therefore, was the potential for bias in the release experiments should some of the ATP on the zinc ore concentrate recovered from the T. ferrooxidans

cultures be non-cell bound. This ATP might have been released as T. ferrooxidans cells lyse during the death phase of a culture or alternatively be released from cells during the trauma of physical/chemical treatments.

Since ATP is a key intermediate in respiratory pathways of the organism and its turnover rate in bacterial cells is stated to be a "matter of seconds" (Lehninger, 1971)), dead cells or cells whose metabolism has been inhibited should rapidly lose their cellular reserve of ATP.

The results of tests of the effect of the two metabolic inhibitors sodium azide and 2,4 - dinitrophenol indicate that both of the inhibitors effectively reduced the ATP content of T. ferrooxidans cultures in 9K medium, i.e. inhibition of the metabolic pathways was achieved (Table III).

The effect of these same metabolic inhibitors upon the ATP content of washed zinc ore particles from a culture of T. ferrooxidans confirmed that a significant amount of the adsorbed ATP was associated with actively metabolizing cells. A substantial reduction of the ATP content was noted by the action of both of these chemical inhibitors as well as by controlled heating at $60^{o}C$ for twenty minutes (Table III). The same inhibitors had only a slight or negligible effect on ATP chemisorbed on zinc ore. Most Probable Number counts performed on washed zinc ore particles confirmed, after a waiting period of 18 days, that the particles contained T. ferrooxidans cells at numbers greater than 10^{5}.

5.5. Evaluation of Release Treatments

5.5.1 Calculation

The results of the screening tests are shown in Tables IV - VII. In our experiments, free cells were defined as those which remained in the supernatant solution following centrifugation at a low speed setting (bench-top centrifuge) for 15 seconds to separate the particles. The measurement of ATP in T. ferrooxidans cultures (without particles) before and after centrifugation at this low speed had indicated that cells remained in the suspension.

TABLE III EFFECT OF METABOLIC INHIBITION ON THE ATP LEVELS OF ZINC ORE/ T. FERROOXIDANS SUBSTRATES AND ATP CHEMISORBED ON TO ZINC ORE

TREATMENT	% REDUCTION OF ATP		
	T. FERROOXIDANS CULTURE IN 9K MEDIUM	ZINC ORE/ T. FERROOXIDANS PARTICLES	ZINC ORE / ATP PARTICLES
900 ppm 2,4-Dinitrophenol 1 hr. @ room temperature	81	62[1]	0
900 ppm Sodium Azide 1 hr. @ room temperature	93	46[2]	10
Heat 60°C - 20 min.	81	62	0

[1] ATP loading on particles - 216 ng/mL

[2] ATP loading on particles - 300 ng/mL

The particle-associated cells following a treatment were defined as the fraction of the ATP which remained with the pellet during the low speed centrifugation. The recovery of ATP refers to the measured cellular ATP remaining in both the supernatant and particle phases following a treatment. It is expressed as the percentage of the original ATP loading on particles. Free ATP, released into solution due to cellular damage during the treatment would not be measured in the post-treatment assays since it would be removed in the filtrate when the cells are transferred to a filter for the extraction of ATP.

Two calculation methods were used to measure the percentage of cells released by a treatment (as indicated by the recovery of ATP in the supernatant solution). In the first approach, the value for the cellular ATP in the supernatant solution was expressed as a percentage of the original ATP loading on the particles, i.e., the absolute value for the recovery of ATP. In the second method, the cell-associated ATP in the supernatant solution was calculated as a percentage of the total cell-associated ATP measured in both particulate and supernatant fractions following the treatment protocol.

The second value for the percentage recovery of ATP was intended to apply a correction for the possible changes of the ATP concentrations of bacterial cells due to trauma during a release treatment. Decreases in ATP content of cells can occur without loss of cell viability. Thus, a treatment protocol which afforded an apparent low total recovery of cell-associated ATP but with a high percentage of the ATP in the supernatant fraction (i.e., released from particles) could deserve more intensive investigation. However, if assessed from the standpoint of only the original ATP loading on particles, a promising release technique might be ignored. The calculation assumes that both released cells and cell remaining attached to particles would have suffered the same degree of trauma and that the ATP content of all bacterial cells would have changed by an equal percentage.

The use of this method of calculation compensates for a lack of knowledge of the effect of the different physical and chemical treatments on the metabolic status and hence the immediate concentration of ATP in the cells. This would have been an extensive study in its own right. For the current screening programme, a high value for the percentage release of cells based on the "recovered" ATP in a test would ensure that a release agent or technique be flagged for additional investigation.

5.5.2 Physical Techniques

Based on the cell-associated ATP released into the supernatants, none of the treatments afforded a satisfactory and reproducible release of viable cells. Additionally, the losses of ATP were high in many instances and this suggested that the cells were experiencing severe trauma. Even allowing for retention of viability in such traumatized cells, the release of cells was poor in almost all cases. (See Table IV).

Based on preliminary tests, homogenization for 15 seconds at full speed (Polytron homogenizer) was selected for the majority of the tests. As additional tests of the respective physical techniques were carried out as controls throughout the test programme, sonication at the lowest instrument setting of 1.0 for 15 seconds also appeared acceptable. However, whereas the sonication gave consistently lower recoveries of ATP, the release of cells appeared to be better than with homogenization. This difference may be attributed in part to the higher biomass (ATP) loadings on the particles used in the sonication tests. Most of the homogenization tests at the beginning of the programme used younger cultures with relatively low biomass loadings as indicated by the ATP values.

In the case of homogenization, the time intervals of 30 - 240 seconds whether at half-speed or full speed, gave consistently lower recoveries of ATP, (Table IV). A similar effect was noticed with the sonication. Factors contributing to these losses of cellular ATP may have included the high shear force, the slight increase in temperature (no cooling was used) or the

TABLE IV COMPARISON OF PHYSICAL TECHNIQUES FOR EFFECTIVENESS OF RELEASE OF CELLULAR ATP FROM OFF ZINC ORE CONCENTRATE

TREATMENT[1]			ATP CONCENTRATION ON UNTREATED PARTICLES (ng/mL)	CELLULAR ATP RELEASED INTO SUPERNATANT		RECOVERY OF ATP (Particles + Supernatant) (% OF ORIGINAL)
				% OF RECOVERED ATP[2]	% OF ORIGINAL ATP	
Shaking by hand		30 sec.	220	1.8	1.2	69
Shaking by hand		30 sec.	610	3.5	1.7	48
Homogenization[3]	- Full Speed,	15 sec.	238	5.4	5.8	108
Homogenization	- Full Speed,	60 sec.	261	0.21	0.09	42
Homogenization	- Full Speed,	120 sec.	261	0.07	0.03	42
Homogenization	- Half Speed,	30 sec.	270	13	7.9	60
Homogenization	- Half Speed,	60 sec.	270	2.7	1.1	42
Homogenization	- Half Speed,	120 sec.	270	1.6	0.75	47
Homogenization	- Half Speed,	240 sec.	270	1.4	0.73	52
Homogenization	- Full Speed,	15 sec.	304	3.8	4.6	120
Homogenization	- Full Speed,	30 sec.	304	7.7	5.5	72
Homogenization	- Full Speed,	15 sec.	n/a[5]	9.7		
Homogenization	- Full Speed,	15 sec.	n/a[5]	18		
Homogenization	- Full Speed,	30 sec.	619	4.0	3.0	74
Homogenization	- Full Speed,	15 sec.	1980	67	40	60
Homogenization	- Full Speed,	15 sec.	2780	14	14	98
Vortex Mixing		30 sec.	619	12	14	119
Vortex Mixing		30 sec.	2280	31	24	78
Vortex Mixing		15 sec.	697	12	12	101
Vortex Mixing		15 sec.	697	12	14	116
Vortex Mixing		30 sec.	697	22	22	101
Vortex Mixing		30 sec.	1980	31	29	108
Sonication[4]	Setting 1.0	15 sec.	419	29	22	77
Sonication	Setting 1.0	15 sec.	419	29	22	74
Sonication	Setting 1.0	30 sec.	419	21	18	87
Sonication	Setting 1.0	30 sec.	419	13	7.0	54
Sonication	Setting 1.0	30 sec.	619	21	13	61
Sonication	Setting 1.0	15 sec.	2280	73	65	89
Sonication	Setting 1.0	15 sec.	1310	21	17	81
Sonication	Setting 1.0	15 sec.	1310	39	31	80
Sonication	Setting 1.0	15 sec.	1310	35	24	69
Sonication	Setting 1.0	15 sec.	1310	29	17	58
Sonication	Setting 1.0	15 sec.	1190	48	33	69
Sonication	Setting 1.0	15 sec.	1190	30	24	80

[1] All tests conducted with particle suspension in basal salts medium

[2] Recovery of ATP if losses of ATP are attributed to cell stress

[3] Polytron Homogenizer Brinkmann Instruments Ltd., PT-10 Head

[4] Branson Sonifier, Model No. S, 125

[5] n/a - not available

abrasive action of the particles. Tests with cultures of T. ferrooxidans in 9K medium (no particulates) indicated negligible loss of ATP with either homogenization at full speed or sonication at the lowest power setting for a period of 15 seconds.

The results of some tests did appear promising but when these procedures were repeated, it was found that the results were not reproducible. Such variations were noted with several of the methods or reagents investigated and these variations were noted both between duplicate tests performed on the same day as well as with experiments repeated on separate days. In Table IV, same day tests can be identified by the identical ATP concentrations on the original untreated particles. Within day variations of replicate tests are exemplified by the sonication tests (Table IV) setting 1.0 for 15 seconds, with an original ATP loading on the particles of 1310 ng ATP/mL. The recovery of ATP in these tests (4 tests) ranged from 58 - 81% with a mean value of 72 $\pm$ 11% (CV = 15.3%) while the percentages for the release into the supernatant of the original ATP on the particles ranged from 17 - 31% with a mean of 22.3 $\pm$ 6.7 (CV = 30%).

Significant variations for between day tests are shown by the values for sonication at the same setting as above (Table IV). In this case over 9 tests, the values for the recovery of ATP ranged from 58 - 89% with a mean of 75 $\pm$ 9.0% (CV = 12%) and the percentages for the release of ATP from particles by the treatment ranged from 17 - 65% with a mean of 28.3 $\pm$ 14.8% (CV = 52%). Homogenization at full speed with particle samples containing ATP loadings of 238, 304, 1980 and 2780 ng ATP/mL (Table IV) gave for the above parameters in four tests, ranges of 60 - 120% for ATP recovery (mean 96.5 $\pm$ 25.9%, CV = 27%) and 5.8 - 40% for percentage release of ATP (mean 16.1 $\pm$ 16.4%, CV = 101%).

All of these tests were conducted with the 9K basal salts medium (no ferrous iron), pH 2.1 as a standard solution. The CV's for these tests were generally significantly greater than noted for the mean CV's for the ATP extraction and analysis technique applied to cell suspensions in liquids or

untreated particle/cell materials (see Section 5.1). Alternative sources of the variations were considered including sampling errors, biomass distribution or biomass loading on particles, age of cultures and variations of technique.

Sampling errors and biomass distribution on particles did not appear to be factors since the identical sub-sampling protocols were used with both the untreated particles and cell suspensions and the particles and supernatants following the release tests. Biomass loading and the age of cultures may have contributed to certain of the between day variations in the percentages of ATP released by a treatment and this will be discussed later. However, they would not explain the discrepancies in CV since the duplicate tests on untreated particles (Section 5.1) were also conducted on particles with different biomass loadings and ages. It was concluded that slight but important modifications of treatment parameters (homogenization times etc.) could be a source of the variations due to the sensitivity of the T. ferrooxidans cells to the physical treatments.

Vigorous shaking by hand caused significant reductions in the recovered ATP. Vortex mixing appeared to be least damaging to the cells but as with other techniques, the release of ATP was generally very low. The consistent low values for the release of ATP (and hence cells) by these physical techniques is in agreement with the observations of other researchers. Abrasive action by the particles may have been detrimental to the cells. Temperature would also be a more significant factor when longer treatment times were used with both homogenization and sonication.

The mechanical homogenization was selected over vortex mixing, since at first sight, it appeared to offer better control of the treatment i.e. timing and speed settings could be more accurately controlled. The same attributes applied to the sonication. However, based on the current results it is not possible to rule out minor variations of the treatment protocols as the source of the poor replicates in release tests.

As noted earlier, the release of cells by these physical techniques was generally low. In one instance, using sonication of zinc ore particles (ATP loading 2280 ng/mL) the percentage release of original ATP loading was 65% but this was not matched by other replicate tests. In most tests where the biomass loading was approximately 2000 ng ATP/mL or higher, the release of ATP from particles was generally better with each of the physical treatments than with lower ATP loadings. The exception to the trend was the most heavily loaded sample (2780 ng ATP/mL) for which homogenization at full speed for 15 seconds released only 14% of the cellular ATP.

Most of the physical treatments' data were generated at regular intervals during the screening of potential release agents. The effect of biomass loading or age of a culture on the ease of release of cells (ATP) only became apparent at a late stage of the experimental programme. At that time, it was discovered that promising releases of ATP by some reagents combined with homogenization or sonication could also be attained by the physical treatment without added reagent. There appears therefore to be a relationship between the nature of the cell/surface association and the average metabolic age of the cells. Older cells may also be more resistant to physical forces extended by the treatment methods.

Culture conditions such as the age of a culture or the size of inoculum used appear to be important factors. Because of the time constraints of the programme, it was not feasible to use cultures of identical age. Similarly, in order to accelerate the generation of urgently needed substrates, the inoculum used in later cultures was greater than in the earlier ones. The higher ATP loadings, which appear to be related to the easier release of ATP by direct physical treatments as noted at later stages of the screening programme, were generally associated with older cultures.

5.5.3 Physical Treatments Combined With Selected Chemical Reagents

The data generated by these screening tests are shown in Tables V - VII. Based on the release of cellular ATP and the overall recovery of ATP, none of the methods afforded satisfactory and reproducible results.

TABLE V EFFECT OF pH ON THE RELEASE OF CELLULAR ATP FROM OFF ZINC ORE PARTICLES

TREATMENT[1]	ATP CONCENTRATION ON UNTREATED PARTICLES (ng/mL)	CELLULAR ATP RECOVERED IN SUPERNATANT		RECOVERY OF ATP (Supernatant + Particles) (% of Original)
		% OF RECOVERED ATP[2]	% OF ORIGINAL ATP	
0.2N HCl , 1 min, no agitation	255	3.8	3.5	91
0.2N HCl , 5 min, no agitation	255	11	4.6	42
0.2N HNO_3 , 1 min, no agitation	255	12	7.9	157
0.2N H_2SO_4 , 1 min, no agitation	255	7.2	7.1	100
0.2N H_3PO_4 , 1 min, no agitation	255	1.1	5.0	452
0.2N H_2SO_4 , Homogenize/Full Speed, 15 sec.	310	24	16	70
0.2N HCl , Homogenize/Full Speed, 15 sec.	310	24	14	57
0.2N HNO_3 , Homogenize/Full Speed, 15 sec.	310	26	18	74
0.2N H_3PO_4 , Homogenize/Full Speed, 15 sec.	310	1.1	0.33	30
0.2N H_3PO_4 , 5% DMSO[3]/shake 60 sec.	216	2.0	1.6	83
0.2N H_2SO_4 , 5% DMSO/shake 60 sec.	216	9.4	8.3	113
0.2N H_2SO_4 , 1M Na_2SO_4/Sonicate 15 sec.	906	35	14	39
0.2N H_2SO_4 , 1M Na_2SO_4/Sonicate 15 sec.	906	33	15	44

[1] Solutions in distilled water

[2] Recovery of ATP if losses of ATP are attributed to cell stress

[3] Dimethylsulphoxide (DMSO)

TABLE VI EFFECT OF DETERGENT SOLUTIONS ON THE RELEASE OF CELLULAR ATP FROM OFF ZINC ORE PARTICLES

TREATMENT[1]	ATP CONCENTRATION ON UNTREATED PARTICLES (ng/mL)	CELLULAR ATP RECOVERED IN SUPERNATANT		RECOVERY OF ATP (Supernatant + Particles) (% of Original)
		% OF RECOVERED ATP[2]	% OF ORIGINAL ATP	
0.001 % C2M[3] /Homogenize	843	30	18	59
0.001 % C2M /Homogenize	232	11	8.6	76
0.001 % C2M /Homogenize	610	16	6.4	40
0.001 % C2M /0.2N H_2SO_4/Homogenize	610	49	55	114
0.005 % C2M /0.2N H_2SO_4/Homogenize	610	22	7.4	34
0.001 % C2M /0.2N H_2SO_4/Homogenize	220	10	6.6	68
0.005 % C2M /0.2N H_2SO_4/Homogenize	220	16	11	68
0.001 % Igepal[4] /0.2N H_2SO_4/Homogenize	220	22	21	103
0.005 % Igepal /0.2N H_2SO_4/Homogenize	220	17	6.5	39
0.001 % Igepal /0.2N H_2SO_4/Homogenize	220	23	18	78
0.001 % Igepal /0.2N H_2SO_4/Homogenize	220	18	13	71
0.001 % Igepal /0.2N H_2SO_4/Homogenize	220	20	9.5	48
0.001 % Igepal / Homogenize	433	30	34	115
0.001 % Igepal / Homogenize	433	28	23	84
0.001 % Igepal / Homogenize	127	24	23	96
0.001 % Igepal / Homogenize	127	20	28	139
0.001 % Tween 80[5] /Homogenize	188	14	13	92
0.01 % Tween 80 /Sonicate 15 sec.	906	34	19	56
0.01 % Tween 80 /Sonicate 15 sec.	906	44	20	46
0.01 % Tween 80 /Sonicate 15 sec.	284	37	25	66
0.01 % Tween 80 /Sonicate 15 sec.	284	40	37	93

[1] All solutions prepared in distilled water. When used, homogenization (Polytron) was for 15 sec. at full speed

[2] Recovery of ATP if losses of ATP are attributed to cell stress

[3] C2M SF Concentrate, amphoteric surfactant, Miranol Co.

[4] Igepal CO 630, non-ionic detergent, (ethoxylated nonyl phenol), Domtar Ltd.

[5] Tween 80, non-ionic detergent (polyoxyethylene sorbitan monooleate), Atlas

TABLE VII EFFECT OF SELECTED CHEMICALS ON THE RELEASE OF CELLULAR ATP FROM OFF ZINC ORE PARTICLES

TREATMENT [1]	ATP CONCENTRATION ON UNTREATED PARTICLES (ng/mL)	CELLULAR ATP RECOVERED IN SUPERNATANT		RECOVERY OF ATP (Supernatant + Particles) (% of Original)
		% OF RECOVERED ATP [2]	% OF ORIGINAL ATP	
1M Ferric chloride / Homogenize 15 sec	188	16	5.9	37
1M Ferric nitrate / " 15 sec	188	19	10	53
1M Copper sulphate / Sonicate 15 sec	830	62	43	69
1M Sodium sulphide/Homogenize 15 sec	232	3.9	1.3	33
0.1 % Ascorbic acid / " 15 sec	232	6.8	4.7	70
0.1M Cysteine / " 15 sec	232	23	18	77
0.1M Dithiothreitol / " 15 sec	232	28	21	77
0.1M Mercaptoethenol/ Sonicate 15 sec	328	42	27	64
0.9M " " 15 sec	328	38	16	43
0.01M Iodoacetamide/Sonicate/Cysteine	1520	65	36	55

[1] Homogenization - 15 sec, Full Speed (Polytron)
Sonication - 15 sec, Setting 1.0 on Sonifier

[2] Recovery of ATP in supernatant if losses of ATP are attributed to cell stress

With some chemical treatments, relatively promising data appeared to be obtained on the initial screening. However, upon repeating the respective tests, often alongside controls of the physical treatment, the release of cellular ATP due to the action of the chemical could not be differentiated from the release of ATP by the physical treatment above. This appeared to be attributable to the effect of the age of the cells on their ease of release.

The tests of the effect of pH and different anions on the release of ATP were predominantly carried out on zinc ore particles with low ATP loadings (Table V). The percentage release of ATP was poor in all cases and did not exceed 18%. Even if the releases were based on the recovered ATP, the data were poor. Sonication and homogenization in the presence of acids gave significantly lower recoveries of ATP. 0.2N HCl was apparently toxic to the cells without agitation. As noted with the physical treatments alone, anomolies were noted which were difficult to explain. The use of 1M sodium sulphate to increase the same strength of the 0.2N H_2SO_4 (pH 1.5) caused a sharp decrease in the recovery of ATP although the results were reproducible. The data would suggest that the cells are sensitive to homogenization at full speed for 15 seconds and that the counter anion had a significant effect on the recovery of ATP. Phosphate + homogenization appeared to be very toxic. The shaking used with 5% dimethylsulphoxide/0.2N H_2SO_4 was much less destructive towards the cellular ATP than was noted with vigorous handshaking in the physical treatments (Table IV). As noted earlier, it appeared that the particles from younger cultures contained more tenaciously bound cells.

The three detergent solutions gave a similar range of data as the other methods (Table VI). The erratic nature of the release data is exemplified by the C2M SF concentrate at a concentration of 0.001% in 2N H_2SO_4 with ATP loadings on particles of 610 and 220 ng/mL respectively. Whereas in the former test the percentage release of ATP was 55%, the duplicate on a different day gave an ATP release of only 6.6%. In the former case the recovery of ATP at 114% was also greater than the 68% noted with the latter sample.

Igepal CO 630 (0.001%) gave more consistent release data when acid was not present and the cells appeared to be relatively unaffected by the detergent.

Tween 80, at 0.01% with sonication also gave reasonably consistent release data although the recoveries of ATP tended to vary more for the replicate tests.

In one test of the effect of 0.01% Tween 80 detergent solution with 15 seconds' sonication on the release of cells, 51% of the original ATP loading on the particulates was displaced. An additional heat treatment of the particles ($60^{o}C$; 20 min.) destroyed a further 38% of the ATP. This indicates that the ATP remaining strongly adhered to the particles was associated with viable cells since ATP chemisorbed on to zinc ore was not affected by heating at that temperature. Based on the overall trend of the repeated tests for all reagents and methods, none of the treatments were successful in releasing this strongly adsorbed fraction. More vigorous physical treatments or more reactive chemicals appeared likely to destroy the cells' viability before attaining their release. As with the other release tests, the higher release with Tween 80 in some tests could be separated from the easier release of cells due to the age factor.

Based on the conflicting data in the literature on the effects of detergent compounds on the dissolution of pyritic minerals by T. ferrooxidans, we limited our choice of detergents to three compounds. Many detergents at low concentrations do not inhibit the iron-oxidizing ability of T. ferrooxidans (Duncan et al, 1964). However, any interference noted with the microbial dissolution of the pyrite minerals was attributed to surface tension/oxygen transfer problems rather than an effect on the attachment of cells to substrate. (Torma et al, 1976).

The selection of chemical reagents for evaluation in our programme was influenced by studies on the association of Thiobacillus thiooxidans with sulphur. The forces/bonds between T. thiooxidans cells and sulphur particles were believed to involve sulphydryl groups on the cell membrane (Takakuwa et al. 1979). This observation was based on evidence that the adsorption of cells to sulphur particles could be prevented by the treatment of cells with compounds able to react with sulphydryl groups. If this was done prior to the addition of the sulphur particles, no adhesion of cells was noted. The blocking effect could be reversed by the addition of an excess concentration of sulphydryl compounds to the cell suspension.

The relative ineffectiveness of the sulphydryl compounds more likely to compete with the cells for binding sites on the ore led us to consider the possible involvement of a metabolic process in the interaction. Takakuwa et al. (1979) had also identified that the ability of T. thiooxidans to adhere to sulphur was affected by the presence of metabolic inhibitors such as 2, 4-dinitrophenol, sodium azide, p-chloromercuribenzoate and iodoacetamide. The sulphur-oxidizing activity was also inhibited and suggested a physiological role in the adhesion process. Anoxia also inhibited the adsorption of cells to sulphur.

The data in Table VII summarize the chemical treatments investigated within the time frame of this study. As noted earlier, chloride ion was toxic to the T. ferrooxidans cells and sodium sulphide (1M) was also not compatible with the cells. Sulphydryl compounds (cysteine, dithiothreitol, mercaptoethanol at concentrations of 0.1 M) gave slightly better release of ATP but not sufficiently high as to be of value. Copper ion (1M) appeared to give a promising release of the ATP but in a duplicate test with a control of the physical treatment (no reagent), the release of ATP by the physical treatment was as high in the absence of the copper ion. Thus the high release was attributed to a change in the nature of the cell/particle association at higher biomass loadings. Ascorbic acid and sodium sulphide (the reducing agents) at concentrations of 1M gave a poor release of cellular ATP.

Tests with sulphydryl reagents were limited to iodoacetamide. In preliminary tests (not reported) with T. ferrooxidans cultures in 9K medium, it was found that the effect of silver (0.01M) and mercury (0.01M) ions on the cellular ATP content was not reversible by the addition of an excess of cysteine. In the case of iodoacetamide, (which was selected because of its solubility at pH 2.3) it was found that the initial decrease in the ATP concentrations to a value of 68% (noted after an incubation period of ten minutes) could apparently be reversed by the addition of an excess (0.5 M) of cysteine. In fact, the addition of cysteine significantly enhanced the recovery of ATP (i.e., 179%) based on the original ATP concentration in untreated 9K cultures.

These data with iodoacetamide were unexpected. As an enzyme inhibitor reacting with sulphydryl groups, iodoacetamide inhibition is usually regarded as an irreversible. Thus, the apparent reversal of the reaction after a brief incubation period of ten minutes was surprising. Still more surprising was the degree to which the cysteine "restored" the ATP level.

When tested on zinc ore/T. ferrooxidans particles (Table VII), the recovery of ATP (55%) after the addition of cysteine was significantly lower although the release of cellular ATP to a level of 36% appeared to show some promise. However, when this test was repeated alongside a control of the physical treatment (sonication) without reagent, no significant difference could be detected between the release of ATP by the reagent and the physical treatment.

6. CONCLUSIONS

In this screening programme, the ATP assay of viable biomass has been used as a rapid technique to monitor the efficiency of various treatment protocols to release viable T. ferrooxidans cells associated with zinc ore particles. Following this screening programme, it was intended that the more promising procedures would be incorporated into enumeration methods based on traditional assay techniques for viable cells such as plate counts.

The ATP method for estimating viable biomass offered a solution to the particular problem relating to this type of method development namely the magnitude of the viable cell population originally associated with particles. This figure is the base from which the success of any release technique will be judged. Similarly, the method must be able to measure the percentage of viable cells released by a treatment, the number of viable cells remaining associated with the particles and hence the overall loss of viable cells due to physical or chemical trauma. This is a nigh impossible task by traditional enumeration methods and while there are still many questions to answer regarding the ATP concentration/viability relationship within cells, the ATP assay appeared to be a novel approach for this programme. In the course of this study, several important variables were identified for which current information is inadequate.

None of the test protocols investigated in this phase of the study gave a satisfactory release of viable cells from zinc ore particles as indicated by the cellular ATP concentrations. Even when the release of ATP was based on the recovered ATP after a treatment, the release of cellular ATP generally did not exceed 50%. It was not therefore feasible to proceed to a traditional enumeration method incorporating one of the release protocols.

Large viable cell populations, e.g., in excess of 10^8 cells/mL of particles, were demonstrated to be associated with zinc ore concentrate in active cultures. The correlation between the estimate of bacterial numbers by the ATP and protein assay techniques was satisfactory in growing cultures of T. ferrooxidans in 9K medium but a signficant discrepancy was noted for the cell estimates by the two methods applied to particles. Although not conclusively proven, the indication is that the protein analyses in the presence of zinc concentrate have a positive bias.

Significant variations noted between the percentage release of cellular ATP in both same day and separate day replicates of release protocols did not appear to be attributable to analytical variations within the ATP assay procedure. Factors believed to contribute to these discrepancies include the age of the culture, the relative loading of biomass and culture variables such as inoculum size which all may influence the percentage of the cells that is bound tenaciously to the particles. A fourth factor would be of slight but important variations of the physical treatment protocols between replicates on the physical/or metabolic stability of the T. ferrooxidans cells.

A fraction of the cellular ATP adheres extremely tenaciously to the particles - particularly in younger cultures. In older cultures or particles with higher loadings of cellular ATP, the percentage of cells that could be more easily dislodged by physical treatments was significantly higher. However, there was still an apparently tightly associated fraction in the older cultures.

It is concluded that ATP assay technique has significant potential for future studies relating to the viability status of particle/cell interactions. However, there are many aspects of the relationship between the ATP concentrations of T. ferrooxidans cells and retained viability that must be better understood. The possibility that the association between Thiobacillus and particles may involve a physiological mechanism emphasizing the need for methods of assessing the viability of cells associated with particles. Deviations between cell estimates calculated from protein and cellular ATP concentrations in cultures or on particulates suggest that the use of protein values as a cell estimating procedure may only be valid with T. ferrooxidans cells in the log phase of growth cycle. However, the observation that free non-cellular ATP could adsorb on to zinc ore particulates at the low pH values used in cultures and tests indicates a potential source of error in using the ATP values to measure the viable cell counts on particles.

This programme has been directed at methodology to enumerate specifically T. ferrooxidans in slurries. This recognizes the key role for this organism in the generation of acid from sulphide minerals. In view of the very tenacious association of certain T. ferrooxidans cells with surfaces, it is concluded that more fundamental investigations are required into the nature of the cell/particle interaction.

The biological generation of acid from sulphide mineral (tailings) remains a concern. A technique is still required to allow an assessment of the populations of acid-generating microorganisms within a tailings pile well in advance of acid leachate being detected. It is concluded that an alternative to the enumeration of T. ferrooxidans may be to seek monitoring techniques not specific for this organism. Thus, the emphasis will be to identify methods to monitor the populations of unspecified acid-generating microorganisms within tailings without requiring the separation of cells from particles.

7. RECOMMENDATIONS

- The tenacious nature of the association that can exist between T. ferrooxidans and the surfaces of sulphide-containing particulates such as zinc concentrate has been further confirmed.

- In order to develop a direct and accurate method to enumerate T. ferrooxidans in slurries based on traditional methods (plate counts), a technique is still required to separate the tightly adsorbed bacteria from the surfaces of particles.

- Future studies should include more fundamental investigations into the nature of the association between T. ferrooxidans and the mineral surfaces.

- Studies should investigate whether or not a physiological mechanism is involved in the bacterial/particle association.

- The ATP assay, to be fully effective as a monitoring technique for the release of T. ferrooxidans cells, requires additional information on the correlation between cellular ATP concentrations and the viability of the organism.

- The feasibility of using non-specific techniques to monitor the biological acid-producing potential of samples from within tailings should be investigated i.e. methods not requiring the release of bacterial cells from particle surfaces.

R.G.W. Laughlin, Ph.D.
Assistant Director
Environmental and Chemical
Engineering Division

J. Christison, Ph.D.
Senior Research Scientist
Environmental and Chemical
Engineering Division

JC/jmm

APPENDIX I

ATP Extraction Methods

A. Cellular ATP in Cultures of Supernatants After Release Treatments

An aliquot (1 mL) of a culture or supernatant suspension was transferred (Clay-Adams pipette) to a filter unit fitted with a GF/F filter (Whatman). Vacuum (250 mm mercury) was applied until the liquid had just broken the surface of the filter. After the vacuum was released, acetone (5 mL) was pipetted on to the cells on the filter unit. The acetone was then removed by re-applying the vacuum. The cells on the filter were then extracted twice with 0.5 mL aliquots of 0.01M sodium triphosphate (Na_3PO_4) buffer pH 11.7. A full vacuum (approximately 600 mm mercury) was maintained on the system throughout this treatment in order to maximize the collection of the ATP extractant.

The ATP extract was diluted with an equal volume of 0.01M Magnesium ethylene diamine tetracetic acid, disodium salt (Mg EDTA). A second dilution (1:1) was made with 0.005M Na_3PO_4/0.005M Mg EDTA buffer solution. Both dilutions were then assayed for ATP by the firefly luciferase method using a Turner Designs, Model 20 photometer. The reaction was initiated by injecting 50µL of luciferin/luciferase reagent (Turner Designs, California) into 50µL of the diluted ATP extract in a cuvette. If the measured ATP contents of the dilutions were not proportional to their dilution factors, further dilutions in the phosphate/Mg EDTA buffer were made and analyzed until proportionality was observed. If sensitivity was a limitation so that inhibitors to the assay method could not be eliminated by dilution, the potential error in the assay procedure was compensated for by using the method of additions to quantitate the ATP present in the sample.

B. Cellular ATP on Particles (Zinc Ore Concentrate)

Particles (from cultures) or following treatments were transferred on to the GF/F filter in the filter unit with basal salts solution (12 mL). Liquid was removed by controlled suction. This was halted as soon as the liquid had passed through the filter and the particles were rinsed with acetone (5 mL). Acetone was removed by suction until the particles and filters appeared to be dry. The filter and particles were transferred to a Na_3PO_4/Mg-EDTA buffer (9 mL) in a scintillation vial. Contents were homogenized (Polytron homogenizer, Brinkmann Instruments, PT-10 head) for 2 minutes at full speed. The homogenate was filtered (0.22 µm membrane filter). Filtrate was then analyzed for ATP as described in Section A.

C. Cellular ATP on Pyrite Particles and in Supernatants from Pyrite Cultures

The methodology was basically the same as for zinc ore concentrate except that at the dilution stage prior to analysis the solutions were diluted with glycine/Mg EDTA buffer.

APPENDIX II

ELEMENTAL DATA ON ZINC ORE SAMPLES*

SAMPLE	ELEMENT / SPECIES CONCENTRATION (% by weight)					
	Fe	Pb	Zn	Cu	S	S_1O_2
A - Zinc Concentrate	17.0	0.6	4.0	0.6	36.5	1.5
B - Flotation Concentrate	18.0	9.0	25.0	1.0		

* Data supplied with samples

APPENDIX III

TEST PROTOCOL FOR RELEASE TREATMENTS ON PARTICLES

A. Sampling and Washing Particles

Cultures of T. ferrooxidans/zinc ore concentrate in basal salts medium was allowed to settle. Supernatant solution was transferred by pipette to another culture flask. Particles were transferred (using a plastic Pasteur pipette) into the barrel of an inverted 1 mL plastic syringe whose tip had been cut off at the 0.1 mL level. The particles were allowed to settle until approximately 0.1 mL had collected on the head of the syringe plunger. The syringe was injected into a paper towel to remove excess liquid. The volume of the "blotted" particles, was adjusted to 0.1 mL.

The sample was ejected into a centrifuge tube containing basal salts solution (12 mL). The tube and its contents was centrifuged for 15 seconds at half speed (Christ, bench top centrifuge). The supernatant was removed with gentle aspiration and the washing with basal salts was repeated twice more. The particles, when drained of liquid, were ready for treatment.

B. Release Treatment on Particles

The particles were transferred to a scintillation vial and suspended in test solution (4 mL). If appropriate a physical treatment (homogenization at full speed or sonication at setting 1.0) was applied, usually for a period of 15 seconds. The homogenate was centrifuged at half speed, 15 seconds and particles and supernatant were each transferred to the filter unit for assay of its cellular ATP content as described in Appendix I.

APPENDIX IV

BIBLIOGRAPHY

Cairns, J.E., S.G. Nutt, B.K. Afghan, and D.B. Carlisle (1979). "Application of the ATP Assay for Monitoring the Disinfection of Wastewater Streams". Environment Canada Scientific Series No. 108. Ministry of Supply and Services Cat. No. En 36-502/108.

Chappelle, E.W., G.L. Picciolo, H.Okrend, R.R. Thomas, J. Demming, and D.A. Nibley (1977). "Significance of the ATP Assay for Characterizing Bacteria". Proc. 2nd Bi-Annual ATP Methodology Symp. San Diego. pp. 611-629.

Duncan, D.W., P.C. Trussell and C.C. Walden (1964). "Leaching of Chalcopyrite with Tiobacillus ferrooxidans: Effect of Surfactants and Shaking." Apl. Microbiol. 12: 122-126.

Hamilton, R.D. and O. Holm-Hansen (1967). "Adenosine Triphosphate Content of Marine Bacteria.". Limnol. Oceanog. 12: 319-324.

Lee, C.C., R.F.Harris, J.K. Syers, and D.E. Armstrong (1971). "Adenosine Triphosphate Content of Selenastium capricornutium". Appl. Microbiol. 21: 957-958.

Lehninger, A.L. (1971). "Bioenergenetics". Second Edition. W.A. Benjamin, Inc., Menlo Park, California.

Lowry O., M. Rosebrough, A. Farr and R. Randall (1967). "Protein Measurement with the Folin Phenol Reagent". J. Biol. Chem. 193: 265-273.

Malley, N. and D.L. Henry (1981). "Advance in Water Quality Monitoring and the Use of Firefly Luciferine/Luciferase/ATP Biossay". Desalination 39: 521-528.

Myerson, A.S. and P. Kline (1983). "The Adsorption of Thiobacillus ferrooxidans on Solid Particles". Biotechnol. Bioeng. 25: 1669-1676.

Proteau, G. and M. Silver (1977). "A Rapid Method for the Determination of Cellular Protein in the Presence of Elemental Sulphur". Can. J. Microbiol. 23 : 1492-1494.

Qureshi, A.A. and J. Patel, (1976). "Adenosine Triphosphate Levels in Microbial Cultures and a Review of the ATP Biomass Estimation Technique." Inland Waters Directorate Scientific Series No. 63, Environment Canada. Ministry of Supply and Services Cat. No. En 36-502/63.

Silverman, M.P. and D.G. Lundgren (1959). "Studies on the Chemolithotrophic Iron Bacterium Ferrobacillus ferrooxidans. I. An Improved Medium and a Harvesting Procedure for Securing High Cell Yields". J. Bacteriol. 77: 642-647.

Takakuwa, S., T. Fujimori, and H. Iwasaki (1979). "Some Properties of Cell-Sulfur Adhesion in Thiobacillus thiooxidans". J. Gen. Microbiol. 25: 21-29.

Tobin, R.S., J.F. Ryan and B.K. Afghan (1978). "An Improved Method for the Determination of Adenosine Triphosphate in Environmental Samples". Water Res.(G.B.) 12: 783-792.

Torma, A.E., G.G. Gabra, R. Guay and M. Silver (1976). "Effects of Surface Active Agents on the Oxidation of Chalcopyrite by Thiobacillus Ferrooxidans." Hydrometallurgy, 1: 301-309.

Final Report P-4488/G-5
Revised May 10th, 1985

METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS IN SLURRIES

DRAFT FOR

FOR

ENERGY, MINES AND RESOURCES, CANADA

BY

J. Christison, Ph.D.
N. Belson
J. Cairns (Consultant)
Environmental and Chemical Engineering Division

Work on this project was conducted under the auspices of the Canada Centre for Mineral and Energy Technology, Energy, Mines and Resources, Canada.

DSS File Number	: 26SQ.23440-3-9138
DSS Contract Serial Number	: OSQ83-00198
CANMET Project Number	: 340212
CANMET Scientific Authority	: Dr. V. Sanmugasunderam

April 26th, 1985

Revised May 10th, 1985

This programme has been directed at methodology to enumerate specifically T. ferrooxidans in slurries. This recognizes the key role for this organism in the generation of acid from sulphide minerals. In view of the very tenacious association of certain T. ferrooxidans cells with surfaces, it is concluded that more fundamental investigations are required into the nature of the cell/particle interaction.

The biological generation of acid from sulphide mineral (tailings) remains a concern. A technique is still required to allow an assessment of the populations of acid-generating microorganisms within a tailings pile well in advance of acid leachate being detected. It is concluded that an alternative to the enumeration of T. ferrooxidans may be to seek monitoring techniques not specific for this organism. Thus, the emphasis will be to identify methods to monitor the populations of unspecified acid-generating microorganisms within tailings without requiring the separation of cells from particles.

7. RECOMMENDATIONS

This programme has investigated the development of improved methodology for the accurate enumeration of T. ferrooxidans in slurries. Experimental studies have concentrated on the ability of chemical/physical treatments to release viable T. ferrooxidans from the surface of sulphide minerals and the ATP assay procedure was introduced as the "best available" method to monitor the efficiency of the various treatments. While no successful release technique has been obtained to date, (which confirms the tenacious nature of the cell/particle interaction), the study has identified very basic problems which must be overcome before a reliable method for the enumeration of T. ferrooxidans in slurries can be used as a standard procedure.

It is, therefore, recommended that the following fundamental studies be conducted to develop sufficient information to develop a reliable, readily usable enumeration method.

Revised May 10th, 1985

1. A fundamental investigation into the nature of the association between T. ferrooxidans and the mineral surfaces.

It has been demonstrated in the literature that particles (both sulphide and non-sulphide containing) inhibit the iron-oxidizing ability of T. ferrooxidans. This information, together with published observations on a related organism, T. thiooxidans, suggests that a physiological mechanism may be involved in the interaction of viable cells of T. ferrooxidans with particles. If so, the concept of using reversible inhibitors of the cells' metabolism may allow the gentle release of cells from particle surfaces. However, successful procedures will require more detailed information on the key metabolic reactions involved and the identification of suitable inhibitors whose effect can be reversed without significant loss of cell viability.

2. Develop a method of separation based on the studies described above which can be routinely used as a technique to separate viable T. ferrooxidans from mineral particles.

This is fundamental if traditional enumeration methods are to be considered (viz: plate counts).

3. Continue to investigate and refine the ATP assay as an aid in method development.

Fundamental studies of the T. ferrooxidans/particle interaction involve the use of pure cultures of the organism. Cell viability is a key parameter and it is considered that the ATP assay has significant potential as an indicator method in such studies.

However, to be fully effective in this capacity or as a monitoring technique for the release of T. ferrooxidans from particles, additional information is required on the correlation between cellular ATP concentrations and the viability of the organism. A major advantage of the technique is its ability to serve as an indicator of the number of viable cells associated with particle surfaces. However, at present, it is not possible to identify whether released ATP originates from a few very active cells or many weakly metabolizing cells. Studies are required to correlate ATP concentrations in cells with cell viability indicated by other techniques.

4. Investigate non-organism-specific techniques of monitoring biological acid-producing potential of tailings samples.

The current study has concentrated on specific methodology for T. ferrooxidans. In environmental situations, the need is for a method to assess the status of biological populations potentially involved in the generation of acid from within sulphide tailings, i.e., to identify changes in such populations and hence developing problems long before symptions have become apparent. T. ferrooxidans has a key role in acid generation which accounts for the present study. However, it is recommended that the feasibility of using non-specific techniques to monitor the acid-producing potential of biological populations within tailings should be investigated. Such methods would not require the release of bacterial cells from particle surfaces. This concept would depend on the use of non-specific measurements of cell viability (such as ATP levels) and studies would be required to correlate such measurements with eventual acid-producing ability.

Revised May 10th, 1985

The efficacy of this approach will depend upon whether a suitable indicator of cell viability can be identified, and whether the "non specific" assay can be made "specific" to acid-producing organisms.

DRAFT FOR DISCUSSION

R.G.W. Laughlin, Ph.D.
Assistant Director
Environmental and Chemical
Engineering Division

J. Christison, Ph.D.
Senior Research Scientist
Environmental and Chemical
Engineering Division

JC/jmm